University of Alberta

Biochemical and Physiological Studies of Saskatoon (*Amelanchier alnifolia* Nutt.) Fruit During Ripening and Storage

by

Suzy Yvonne Rogiers ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of *Doctor Of Philosophy*

in

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For Sergio

Abstract

This work characterizes the ripening physiology and postharvest behavior of saskatoon fruit. The efficacy of controlled atmosphere storage for slowing physiological and pathological deterioration of fruit was evaluated. A physicochemical characterization of saskatoons lead to the development of a nine stage maturity index system. The major carbohydrates in fruit were glucose, fructose and sorbitol, all of which increased during ripening. Succinate and malate were the predominant organic acids in immature and mature fruit, respectively.

Fruit ethylene content and ethylene evolution increased during ripening. Aminocyclopropane-1-carboxylic acid (ACC) application to fruit developing on the plant hastened ripening, while application of inhibitors of ethylene synthesis delayed ripening. Ethylene synthesis in preclimacteric fruit was limited by ACC synthase and not ACC oxidase. Preharvest and postharvest changes in ethylene production during ripening differed but were consistent with that of climacteric fruit. Fruit displayed a respiratory climacteric on a whole-fruit basis while ripening on the plant.

An increase in oxidative metabolism accompanied ripening. The double bond index of polar lipid fatty acids fell as fruit developed from green to purple, reflecting a progressive increase in the saturation of membrane lipids. Products of lipid peroxidation, ethane and 2-thiobarbituric acid reactive substances increased in fruit during ripening. Lipoxygenase activity also increased, while superoxide dismutase and catalase activities decreased with development. Oxidized glutathione increased as a percentage of total during ripening. Glutathione reductase and transferase activities rose sharply during the final stages of ripening in response to the increasing oxidative stress.

Storage at 0.5°C was more effective than 4.0°C at slowing deterioration of ripe saskatoon fruit. A 2% O_2 atmosphere reduced ethylene production and respiration of nearly ripe to fully-ripe fruit. Of six atmospheres (0.035% and 5% CO_2 with 2%, 10%, or 21% O_2), 5% CO_2 with 21 or 10% O_2 were the most effective at maintaining fruit soluble solids, anthocyanins, firmness and fresh weight over eight weeks of storage at 0.5°C. Storage of fruit in 0.035% CO_2 and 21 or 10% O_2 resulted in the highest titratable acidity, lowest pH, and lowest ethanol concentrations. The high CO_2 atmosphere eliminated fungal colonization of fruit in all three O_2 treatments for at least eight weeks.

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List of Abbreviations

ACC	1-aminocyclopropane-1-carboxylic acid
AOA	α-aminoisobutyric acid
AVG	aminoethoxyvinylglycine
BCA	bicinchoninic acid (4,4-dicarboxy-2,2-biquinoline)
BCIP	5-bromo-4-chloro-indolyl-phosphate
CA	controlled atmosphere
CAT	catalase
DBI	double bond index
CDNB	1-chloro-2,4-dinitrobenzene
DTNB	5,5'-dithio-bis(2-nitrobenzoic acid)
DTT	DL-dithiothreitol
EDTA	ethylenediaminetetraacetic acid
GRase	glutathione reductase
GSH	reduced glutathione
GSSG	oxidized glutathione
GTase	glutathione transferase
LOX	lipoxygenase
met	L-methionine
NADPH	nicotinamide adenine dinucleotide phosphate
NBT	nitroblue tetrazolium
PBS	phosphate buffer saline
PMSF	phenylmethylsulfonyl fluoride
POX	peroxidase
PUFA	polyunsaturated fatty acid
PVPP	polyvinylpolypyrrolidone
SAM	S-adenosylmethionine
SOD	superoxide dismutase
TA	titratable acidity
TBA	2-thiobarbituric acid
TBARS	2-thiobarbituric acid reactive substances
TCA	trichloroacetic acid
TNB	5-thio-2-nitrobenzoic acid

Chapter I

Introduction

The saskatoon (*Amelanchier alnifolia* Nutt.; Maloideae) is native to the Canadian prairies and Northern plains of the United States (Harris 1972). Saskatoon plants are shrubs that range in height from 1 to 7 m. Plants produce white flowers that are held in racemelike determinate inflorescences (Steeves and Steeves 1990), forming trusses that typically yield anywhere from three to eighteen fruit. The fruit is a miniature pome that develops from a gynoecial hypanthium (Steeves et al. 1991), with a maximum diameter of approximately 1.5 cm depending on cultivar and growing conditions, and is most often purple at maturity.

Excellent culinary attributes of fresh and processed fruit, along with a high degree of cold hardiness exhibited by this species, have made saskatoon an ideal fruit crop for the Canadian prairies. Relative to other horticultural crops, total acreage is small (about 1100 ha across the Prairie provinces in 1996), reflecting the infancy of the industry. However, annual distributions of 250,000 to 300,000 seedlings to growers from one commercial nursery in Alberta over each of the last two growing seasons (L. Hausher, 1996, Alberta Agriculture, Brooks, Alberta; personal communication) reflects a high degree of interest in producing the crop, and is evidence of rapid expansion ongoing in this industry. At least part of the production interest has been spurred by the economic potential of this crop. St. Pierre (1992) reported returns per ha ranging from $8,860 to $67,500. Moreover, demand for saskatoon fruit currently exceeds supply (St. Pierre 1992; Hausher, 1996 personal communication). The fruit is consumed fresh, mainly from 'U-pick' operations, or is machine-harvested and processed into value-added products such as jams, pies, liqueurs, etc.

Although there is current demand for saskatoon fruit as a fresh product at the retail level, lack of adequate transport, storage, and packaging systems for preservation of fresh fruit quality have impeded development of a fresh market industry. Historically, development of storage and packaging systems for small fruits (blueberries, raspberries, strawberries, cherries, etc.) has had a positive impact on the production and retail industries, extending marketing period and increasing the demand for fresh fruit. An investigation on suitable methods for storage of fresh saskatoon fruit will thus facilitate expansion of the industry into the retail market. Research on the ripening physiology, biochemistry and postharvest behavior of saskatoon fruit, however, is minimal. Understanding the metabolic bases for ripening and subsequent deteriorative changes in harvested fruits is prerequisite to defining storage systems that will best preserve quality. This work addresses two areas of postharvest research that will facilitate development of a fresh-market industry for saskatoon fruit: ripening physiology and controlled atmosphere storage.

Fruit can be classified as 'climacteric' or 'nonclimacteric', according to their respiratory and ethylene responses during ripening. In nonclimacteric fruits (e.g. cherry, citrus, blueberry, grape, etc.) changes associated with ripening (color, flavor, texture, aroma, etc.) occur relatively slowly and respiration rate declines throughout fruit development (including ripening). In contrast, respiration of climacteric fruits (e.g. raspberry, apple, pear, avocado, banana, etc.) declines until physiological maturity is reached, increases substantially during ripening, and then decreases as maturation proceeds and the fruit eventually senesce. Physiological maturity is thus a stage of development just prior to ripening, where fruits will continue ontogeny even if harvested. It is important to recognize physiological maturity for climacteric fruits, as harvest at this stage results in maximum storage life and the ability to ripen normally when presented with appropriate environmental conditions. Increased respiration of climacteric fruits likely provides the energy (ATP) required to fuel ripening metabolism (e.g. chlorophyll breakdown, synthesis of pigments, carbohydrate interconversions, etc.) (Salunkhe and Desai, 1984).

Ripening of climacteric fruits is controlled by ethylene. Fruit ethylene production is very low (virtually undetectable) prior to the climacteric. Soon after the attainment of physiological maturity, a burst of ethylene production by the fruit triggers the respiratory climacteric and induces metabolic pathways responsible for the many changes that we associate with ripening (Abeles et al., 1992). As a gas, ethylene not only triggers ripening of climacteric fruits but also induces uniform ripening throughout the sphere of the fruit. If saskatoon fruits are climacteric, storage and packaging systems can be designed to inhibit ethylene production, effectively delaying the respiratory climacteric and thus ripening. Low O_2/high CO_2 controlled atmosphere and hypobaric storage, and modified atmosphere packaging systems are extremely effective at inhibiting ripening of climacteric fruits. Low O_2/high CO_2 atmospheres inhibit fruit respiration, ethylene biosynthesis and ethylene action, thereby delaying onset of the climacteric and thus ripening.

An underlying feature of aging and senescence in plants (and animals) is the macromolecular rearrangement of membrane systems in all parts of the cell, which affects reduced stability of various enzyme systems, effectively disrupting metabolism (Leshem, 1987). Deesterification of the sn-2 acyl bond of membrane phospholipids releases polyunsaturated fatty acids which are metabolized by lipoxygenase into hydroperoxides and oxy-free radicals (Leshem, 1987). The resulting lipid peroxidation leads to an increase in saturation of membrane phospholipids, which results in increased gel-phase domains (Fobel et al., 1987), decreased fluidity and increased permeability (Pauls and Thompson, 1980; 1981). One mechanism by which plants defend against the deleterious effects of free radicals is by the induction of superoxide dismutase, which catalyzes the dismutation of superoxide-radicals to O_2 and H_2O_2 (Hassan and Scandalios, 1990). Peroxidase and catalase then catabolize H_2O_2 to H_2O and O_2. By eliminating H_2O_2 accumulation, peroxidase and catalase prevent the further formation of potent free radicals. The activities of these free radical scavenging enzymes usually decrease during the latter stages of plant senescence (Dhindsa, et al., 1981), which contributes to an ever increasing rate of deterioration. Equating the process of ripening with senescence is controversial. However, if similar mechanisms are invoked during ripening of saskatoon fruit then low O_2 atmospheres should be effective at slowing deterioration, simply by limiting the major substrate (O_2) available for oxidative catabolism. Low O_2 atmospheres may thus be an effective adjunct to cold storage through inhibiting the oxidative deterioration of harvested fruit.

This work examines and characterizes the metabolic basis for ripening of saskatoon fruit. In addition, storage systems that slow these processes are tested for their efficacy in maintaining fresh fruit quality. Specific objectives include:

1. Develop a maturity class index system for saskatoon fruit based on the predominant physicochemical changes that occur during maturation and ripening.

2. Determine the role of ethylene in the regulation of saskatoon fruit ripening.

3. Determine the extent to which oxidative metabolism changes during maturation and ripening of saskatoon fruit.

4. Evaluate the efficacy of controlled atmosphere storage for slowing physiological and pathological deterioration.

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Chapter II[1]

Physical and Chemical Changes During Growth, Maturation and Ripening of Saskatoon (*Amelanchier alnifolia* Nutt.) Fruit

Abstract

Both ongoing and future studies of saskatoon fruit would benefit from a maturity index that could be referred to as as an indicator of fruit development. Accordingly, two cultivars of saskatoon fruit ('Northline' and 'Smoky') were sorted subjectively into nine maturity and ripeness stages based on differences in size and color. The physicochemical attributes of each stage were then characterized in detail. Color ranged from mostly green (stages 1-4) to white-pink (5) to pink (6) to red (7-8) to purple (9). The fastest gains in fresh and dry weights occurred over the latter stages of development (from stages 5 to 9), while increases in fruit diameter between stages 1 and 9 were linear. Fruit firmness declined substantially between the first and the fifth maturity stages and then only slightly as ripening progressed to maturity class 9. Fruit nitrogen concentration decreased throughout development as did pH, chlorophyll, and phenolics. Fruit soluble solids, the ratio of soluble solids to titratable acidity, and anthocyanins increased from the fourth to the ninth stage of development. Glucose and fructose concentrations were about equal, increased about 8-fold from class 4 to 9, and along with sorbitol were the major carbohydrates in fruit. Starch was nondetectable in fruit at all stages. Succinate was the predominant organic acid in immature fruit; however, levels declined with advancing maturity and malate was the most concentrated organic acid in mature fruit. Changes in the relative concentrations of quinate, galacturonate, citrate, pyruvate, cis-aconitate, fumarate, and oxalate were also characterized with advancing maturity. In total, the concentration of organic acids dropped at least 2-fold as fruits developed from class 1 to 3, then declined relatively slowly through the remaining developmental stages. Cultivar variability in physicochemical attributes was relatively small and insignificant with regard to sorting and classifying fruits according to maturity. It is proposed that this nine-point maturity index be adopted for future studies on saskatoon fruit growth and development.

[1] Suzy Y. Rogiers and N. Richard Knowles (1997) Physical and Chemical Changes During Growth, Maturation and Ripening of Saskatoon (*Amelanchier alnifolia* Nutt.) Fruit. Can J Bot (in press)

Introduction

The growing interest in commercialization of saskatoon (*Amelanchier alnifolia* Nutt., also known as serviceberry) fruit has resulted in increased research on various aspects of production, handling and processing. Studies on the control of insect and microbial pests (Lange and Bains 1995, Pluim et al. 1994), use of growth regulators to promote uniform fruit development and ripening (McGarry 1996), changes in the chemistry of cuticular waxes during development (Knowles and Knowles 1996), and postharvest physiology and storability of fresh fruit (within our lab) are some of the projects currently underway in Alberta. It became evident early in our postharvest physiology studies that both ongoing and future research would benefit from a maturity index that could be used as a subjective measure of fruit development. Analysis of the physicochemical attributes of each maturity level would increase the value and usefulness of such an index. Changes in organic acid and sugar composition of saskatoon fruit have been characterized toward the end of development (Wolf and Wood 1971); however, the usefulness of this data are limited because qualitative maturity characteristics of the fruit were not described. Changes in anthocyanins, phenolics, carbohydrates, acidity, and colour have mainly been reported from the latter stages of ripening (Green and Mazza 1986). This paper reports the first comprehensive physicochemical analysis of saskatoon fruit development using a nine-class index of maturity, from immature to fully mature and ripe, based on differences in size and color. Fruit at each of the nine developmental stages were further characterized quantitatively by physical (fruit softening, color, size, etc.) and biochemical (pigments, carbohydrates, protein, organic acids, etc.) attributes. Although there are small variations between cultivars, we propose that these nine stages provide a quick reference maturity class index for developing saskatoon fruit.

Materials and methods

Fruit growth and maturity classes

Saskatoon fruit (*Amelanchier alnifolia* Nutt. cvs. Northline and Smoky) were hand harvested from the Grove Berry Patch, Spruce Grove, Alberta, at weekly intervals (approximately 2 kg per harvest) during the 1993 growing season. The fruit from each harvest were transported on ice from the field to the laboratory, where fresh weights of 50 individual fruit were determined. Fruit volume of each of the fifty fruits was calculated with the assumption that a fruit most closely resembled an oblate ellipsoid ($V = 4/3\pi a^2 b$; a = major axis radius, b = minor axis radius). The percentage of fruit falling into any one of nine different maturity classes (see below) was determined at each harvest and plotted against accumulating degree-days (above a base of 4.4°C, McGarry 1996) throughout the growing season.

Fruit were sorted subjectively into nine maturity classes (Fig. 2-1) based on the following criteria. Fruit of maturity classes 1 to 4 were relatively hard and green, except for some pink coloration on the surface exposed to sun, and were sorted visually based mostly on differences in size (volume). Fruit of maturity classes 5 to 9 were sorted on the basis of color differences. This was possible because the green portions of class 4 fruit had faded noticeably to whitish-yellow as they developed to become class 5. Furthermore, the surface of maturity class 5 fruit was at least 50% pinkish-red. Class 6 fruit were light red and further development was reflected in changes to dark red (class 7), dark red with purple hues (class 8) and finally to purple for class 9. While not quantified, it is worth noting that maturity classes 1 to 3 required considerable force to detach fruit from their pedicels during harvesting. Fruit was more easily detached as maturation progressed from class 4 to class 9. These sorting criteria were easily applied to the four test cultivars of saskatoon (Fig. 2-1) and worked equally well with other cultivars and seedling selections (data not shown).

Physical characterization

Surface color of fresh fruit from each maturity class (three replicates, 20 g each) was measured with a Hunterlab model D25 M/L-2 Colorimeter (Hunter Associates Laboratory Inc., Fairfax, VA) standardized to a pink tile. Hunter 'a' values ranged from green (negative) to red (positive), 'b' values ranged from blue (lower numbers) to yellow (higher numbers), and 'L' values described the degree of lightness, from white (higher numbers) to black (lower numbers). Fruit firmness was measured with an Instron Universal Testing Machine, model 4201 (Instron Corp., Canton, MA), equipped with a 500 kg load cell for maturity classes 1-4 and a 50 kg load cell for maturity classes 5-9. The downspeed of the crosshead was 150 mm min^{-1}. From each maturity class, three replicates of fresh fruit (15 g each) were placed individually in a Kramer Shear Compression Cell (Instron Corp.), adjusted by the insertion of two metal plates so that the bottom area was 14 cm^2 (Gill et al. 1979). The return gauge was set so that the compression cycle terminated as the blades emerged from the bottom of the test cell. Fruit firmness was expressed as the force (kg g^{-1} fresh weight) required to penetrate a sample.

Chemical characterization

Chlorophyll was extracted from three replicates (2.5 g each) of frozen fruit with 10 mL of 80% (v/v) acetone at 4°C using a polytron tissue homogenizer (Brinkmann, Rexdale, Ont., Canada). After centrifuging (4500 g, 10 min.) the pellet was washed (4 times) with 5 mL aliquots of 80% acetone to extract the remaining chlorophyll. Absorbance of the combined supernatants was determined at 649 and 665 nm, and total chlorophyll was calculated according to Vernon (1960).

Anthocyanins and phenols were extracted from three replicates of 5 g of frozen fruit with 10 mL of 95% (v/v) ethanol:1.5 M HCl (85:15 v/v) at 4°C using a polytron. Extracts were then centrifuged at 14500 g for 15 min. and pellets were washed twice with 5 mL of the extraction medium. The supernatants were combined and diluted to 25 mL with extraction medium. Total anthocyanins were assayed by the pH differential method of Fuleki and Francis (1968) and were calculated using the extinction coefficient for cyanidin-3-galactoside ($\varepsilon^{1\%}$ 510 nm = 765), the major anthocyanin of saskatoon fruit (Mazza, 1986). Phenolics in the fruit extracts were assayed with Folin & Ciocalteu's phenol reagent (Sigma, Mississauga, Ont., Canada) by the method of Singleton and Rossi (1965). Samples were incubated at 25°C for 15 min and A_{765} was compared with that of a ferulic acid standard curve. Phenolics were thus expressed as ferulic acid equivalents.

Titratable acidity (TA) and pH were analyzed by the electrode method (AOAC 1995). Three replicates of frozen fruit (10 g each) were extracted at room temperature with 100 mL of distilled water using a polytron (1 min.). The extracts were boiled for 30 min., cooled, adjusted to 100 mL, centrifuged at 14500 g (15 min.) and pH of the supernatant was recorded. Titratable acidity was measured by titrating the supernatant to pH 8.1 with 0.1 M NaOH, and was expressed as milliequivalents of NaOH per 100 g of fruit fresh weight.

To determine soluble solids, three replicates of fruit (3 g each) were ground with mortar and pestle at room temperature and extracts were filtered through a 63 µm nylon mesh. The filtrates were centrifuged for 15 min. (1640 g) and the refractive index of each supernatant was measured with an ATAGO hand refractometer (ATAGO Co., Ltd., Tokyo, Japan). The refractometer was calibrated with 0% and 10% sucrose solutions, and refractive index values were adjusted to 20°C.

Sugars and organic acids were analyzed by HPLC using methods modified from Scott et al. (1991) and Coppola and Starr (1986). Three replicates of frozen fruit (5 g each) were extracted at room temperature with 25 mL of 80% (v/v) ethanol. Extracts were incubated for 45 minutes at 75°C and centrifuged at 14500 g for 20 min. The volumes were adjusted to 25 mL and 2 mL of each supernatant was reduced to dryness using a Savant AES2000 Automatic Environmental SpeedVac (Savant Instruments, Inc., Farmingdale, NY, USA). The residue was dissolved in 2 mL of deionized H_2O and filtered through a preconditioned SEP-PAK C_{18} cartridge (Waters

Associates, Inc., Milford, MA, USA), then through a 0.45 µm membrane. The samples were diluted 5-fold with H_2O (HPLC grade) prior to HPLC analysis. Organic acids were separated by passage through an Aminex HPX-87H cation exchange column fitted with a cation H^+ Microguard Precolumn (Bio-Rad Laboratories, Mississauga, Ontario) and were quantified with a Bio-Rad UV monitor (model 1305) set at 210 nm. The column temperature and flow rate were 65°C and 0.4 mL min^{-1}, respectively. The mobile phase consisted of 2.25 mM H_2SO_4. Quinate was resolved from fructose by operating the system at 30°C. All peaks were identified, and total organic acids was calculated as the sum of the individual acids. Sugars were separated by passage through an Aminex HPX-87C column at 85°C and were quantified with a Shimadzu RID-6A refractive index detector (Shimadzu Corp., Kyoto, Japan). The mobile phase was HPLC grade H_2O flowing at 0.7 mL min^{-1}.

In preparation for nitrogen analysis, 100 g of fruit of each maturity class was oven dried (50°C, 5 days) and ground with a Wiley mill (Bodine Electric Comp., Chicago, IL) to pass a 40 mesh screen. Total nitrogen was analyzed by combusting 1 g samples of the ground fruit (2 replicates from each class) in a Leco FP-2000 Nitrogen/Protein Analyzer (LECO® Corporation, St. Joseph, MI, USA).

Results and discussion

Year-to-year trends in growth of saskatoon fruit were more highly correlated with accumulating degree-days than with time (McGarry, 1996). Hence, fruit growth through the 1993 growing season was plotted as a function of degree-days. During this particular season, degree-days increased at a constant rate of 10.7 heat units/day (Fig. 2-2, inset). Saskatoon flowers (cv. Smoky) had completed petal fall by the 300 th degree-day and fruit had developed to maturity class 1 (see Fig. 2-1) in an additional 150 degree-days (14 days). Subsequently, gain in fruit fresh weight was linear in two periods, increasing by 0.35 mg per degree-day between 443 and 718 degree-days, and by 3.40 mg per degree-day between 718 and 940 degree-days (Fig. 2-2). A 10-fold faster growth rate was thus evident over the latter stages of fruit development (from maturity classes 4 to 9). McGarry (1996) showed that an increase in cell number in the mesocarp contributed substantially to saskatoon fruit growth early in development (up to 300-400 degree-days), but mitosis was minimal through the remainder of development. Increases in fruit size beyond about 400 degree-days were manifested primarily by expansion of existing cells (McGarry 1996, Olson and Steeves 1982).

Trends in fruit volume were similar to those for fruit weight (Fig. 2-2). Growth in volume of stone fruits, grapes and blueberries is best described by double sigmoidal trends, while changes in volume of apple, pear, and strawberry fruit are typically described by single sigmoidal trends (Coombe 1976). The slight lag in cumulative volume of saskatoon fruit between 750 and 850 degree-days suggests a double sigmoidal growth pattern in this yield component; however, more data are needed to define the trend adequately. Fruit fresh weight of cv. Smoky reached 0.99 g, and volume reached 0.65 cm^3 before senescence began (Fig. 2-2).

At 443 degree-days fruit were of maturity class 1, and by 650 degree days fruit had developed almost entirely to maturity class 4 (Fig. 2-2; maturity classes 1 to 4 were combined for clarity). The first fruit of maturity classes 5 and 6 appeared 70 degree-days (1 week) later, and fruit of maturity classes 7, 8, and 9 were present after an additional 60 degree-days (5-6 days). Ninety percent of the fruit were fully mature after 1040 degree-days; therefore, about 600 degree-days (8 weeks during this season) were required for fruit to develop from maturity class 1 to 9. An additional 3 weeks (225 degree-days) were required for fruit to develop from anthesis to maturity class 1. The time interval from anthesis to ripeness ranges anywhere from 3 weeks (strawberry) to 60 weeks (orange), with many fruits requiring 15 weeks (Coombe 1976). Thus the total time required for saskatoon fruit to develop from anthesis to full ripeness (maturity 9) is relatively short, at 11 weeks (about 825 degree-days).

At 850 degree-days, 25% of the fruit were of maturity classes 1 to 4, 20% were 5 to 6, 25% were 7 to 8, and 30% were of maturity class 9. The optimum time to harvest fruit with even yield distribution among all of the maturity classes for cv. Smoky is thus about 8 weeks (550 degree-days) from petal drop. By 950 degree-days, 50 % of the fruit had developed to maturity class 9, while the remaining fruit were distributed among the various immature stages. This nonuniformity in ripening is not conducive for once-over machine harvesting. Furthermore, a significant proportion of fruit may be over-ripe by the time a maximum number of fruit have reached optimum ripeness. The potential use of growth regulators to enhance more uniform development and ripening, to facilitate once-over mechanical harvesting, was investigated by McGarry (1996). Exogenous gibberellins did not affect saskatoon fruit set consistently, however saskatoon shrubs treated with ethephon (Ethrel, Amchem Products, Inc., Ambler, PA, USA) prior to harvest yielded greater proportions of ripe fruit in a single harvest. While not desirable for mechanical harvesting, the extended period of mature fruit availability afforded by nonuniform ripening is a favorable characteristic for the U-pick industry.

Trends in fruit growth were somewhat different when plotted as a function of maturity class, which is no doubt a consequence of the variation in time intervals (degree-days) needed to develop each of the nine classes. Accumulation of fresh and dry weights with advancing maturity of 'Northline' and 'Smoky' fruit is best described by cubic polynomials, with the largest increases occurring between maturity classes 6 and 9 (Fig. 2-3). The large gains in fresh and dry weights during the later stages of fruit development have significant implications for harvesting time. Many climacteric fruits (e.g. apple, banana, tomato) are harvested at physiological maturity, before ripening has started, which allows greater time for transport and results in extended storage and shelf life. Most of these fruits attain full size coincident with physiological maturity, well before the changes in color and flavour associated with ripening. We have determined that fruit of maturity classes 6 through 8 will continue to ripen if detached from the plant (data not shown). However, the fact that fruit continue to gain weight at a relatively high rate through class 9 (Figs. 2-1 and 2-3) likely precludes harvesting at developmental stages less than class 9.

'Northline' fruit had 26% more fresh weight, and 20% more dry weight than 'Smoky' fruit at maturity (Fig. 2-3). Dry weight, as a percentage of fresh weight, declined from 31% to 22% as fruits matured from class 1 to 7 (Fig. 2-3, inset), reflecting the rapid rate of water accumulation over these stages. However, as fruit matured beyond class 7, the rate of percent dry weight decline slowed by 70%, indicating less water uptake and/or greater assimilate deposition during this later phase of development. While weight increases with advancing maturity were best described by cubic polynomials, fruit diameter increased linearly by 0.61 mm per maturity class (averaged over cultivar; diameter = 0.61(maturity class) + 6.5, r^2=0.99), and the total increase from stage 1 to 9 was 1.7-fold. 'Northline' fruit attained a mean diameter of 12.3 mm at maturity, while mature 'Smoky' fruit averaged 11.2 mm in diameter (Fig. 2-3). On average, fruit firmness declined about 6.5-fold as fruit developed from class 1 to 5, and then by only 40% during development to class 9 (Fig. 2-3).

Trends in Hunter 'L', 'a', and 'b' values (Fig. 2-4) were consistent with the color changes observed during fruit development (see Fig. 2-1). Hunter 'L' readings for 'Northline' and 'Smoky' fruit increased marginally from maturity classes 1 to 4, then declined substantially as color darkened with further development to maturity class 9 (Fig. 2-4). Hunter 'a' values increased over 15-fold between maturity classes 4 and 6, then decreased beyond maturity class 7 as the degree of 'redness' declined. Hunter 'b' values remained stable through maturity class 4, then 'blueness' increased, resulting in a decline in 'b' values for both cultivars.

Changes in fruit color are often brought about by a loss of chlorophyll in conjunction with the biosynthesis of anthocyanins and carotenoids. Some cultivars of pome fruits, however, do not change color upon ripening and remain green. The color changes associated with maturation and ripening of saskatoon fruits (Figs. 2-1 and 2-4) involved both chlorophyll degradation and anthocyanin biosynthesis (Fig. 2-5). Fruit of cvs. Northline and Smoky contained similar concentrations of chlorophyll and anthocyanins at any particular developmental stage. During development chlorophyll levels declined sigmoidally (about 5-fold in total), with the greatest loss occurring as fruit matured from class 4 to 5 (Fig. 2-5). Chlorophyll loss was due to the simultaneous disappearance of both chlorophylls a and b. Anthocyanin concentrations remained relatively low and constant as fruit developed from class 1 to 5, then increased 6-fold and 7-fold through class 9 for 'Smoky' and 'Northline', respectively. 'Northline' fruit had a 17% higher concentration of anthocyanins at class 9 than 'Smoky' fruit (Fig. 2-5). The anthocyanins in immature saskatoons were mainly concentrated on the side of the fruit exposed to sun (see Fig. 2-1). This phenomenon is common in fruit and is most often related to exposure to UV light which stimulates the *de novo* synthesis of enzymes involved in anthocyanin production (Tucker 1993). The major anthocyanin of saskatoon fruit is cyanidin-3-galactoside, which accounted for 61% of the total anthocyanins in mature fruit of 'Smoky' and 'Honeywood' (Mazza 1986). High fruit anthocyanin concentration is a desirable characteristic for fresh and processed products such as jams, pie fillings and juices, and 'Northline' fruit were therefore of higher quality in this attribute.

As fruit developed from maturity class 1 to 9, total phenolic concentrations declined by half for both cultivars (Fig. 2-5). Phenolic compounds contribute significantly to fruit colour and flavour. Phenols are also partly responsible for the astringent nature of fruits (Tucker 1993). Declining phenolic levels with increasing maturity of saskatoon fruit may contribute to a loss of astringency during ripening. A similar decrease in phenols has been observed with increasing maturity of bananas and persimmons; however, an increase in total phenols occurred during development of peaches and plums (Goldstein and Swain 1963). Many plant phenolics function in deterring colonization by pathogenic fungi and bacteria, as well as insect attack (Agrios 1978). Further research to characterize and correlate changes in susceptibility of saskatoon fruit to pathogens and insect predation, in light of the declining phenolic levels, would be of interest.

Fruit pH declined during development in both cultivars, from 4.8 at class 1 to 4.2 at class 7 (Fig. 2-6). Subsequently, pH remained relatively constant through to maturity class 9 in 'Northline' fruit, but increased to 4.4 in 'Smoky' fruit. TA of 'Northline' fruit was about double that of 'Smoky' fruit throughout development (Fig. 2-6). A maximum in TA occurred at classes 7 and 5 for 'Smoky' and 'Northline' fruit, respectively, and this was followed by a slight decline as fruit of both cultivars developed to full maturity. Similarly, the TA of maturing apples increased during growth and then declined upon ripening (Krotkov 1960). Few studies have characterized changes in the acidity of soft fruits during development; however, total acidity of strawberry increased to the mature green stage and then declined (Spayd and Morris 1981). Acids not only contribute to flavor directly, but are important in the saskatoon processing industry as they affect the gelling properties of pectin (Pilnik and Voragen 1970) and influence color of pigments (Timberlake 1981) in the final product. It has been reported that saskatoon fruits do not possess sufficient natural acidity for satisfactory processing into jams, pie fillings, and wine (Wolfe and Wood 1971); therefore, the higher titratable acidity of 'Northline' fruit probably makes it more suitable than 'Smoky' fruit for processing.

Soluble solids content was relatively low and constant during early development of fruit from both cultivars, and 'Northline' fruit had more soluble solids than 'Smoky' fruit throughout the green maturity classes (Fig. 2-6). As ripening progressed, soluble solids increased faster in 'Smoky' than in 'Northline' fruit. By class 7, soluble solids content of 'Smoky' fruit had surpassed that of 'Northline' fruit and, with further development to full maturity, reached 16.4 °Brix in 'Smoky' fruit and 14.0 °Brix in 'Northline' fruit. The soluble solids/titratable acidity (SS/TA) ratio was about 2-fold greater in 'Smoky' fruit than in 'Northline' fruit at all maturity classes (Fig. 2-6 inset). This was due primarily to the lower TA of 'Smoky' fruit. In fruit of both cultivars, SS/TA remained relatively constant to maturity class 5, then increased as fruits developed to class 9. Sugar:acid ratios are a major contributor to flavor of many fruits (Ulrich 1970) and the values reported here may potentially be used as a measure of saskatoon fruit maturity and ripeness. The ratios for maturity classes 7, 8, and 9 are consistent with those reported by Green and Mazza (1986).

Glucose, fructose, and sorbitol were the most abundant sugars in ripe saskatoon fruit (Fig. 2-7), together accounting for 99% of the total sugars. In immature fruit, these sugars were all within a concentration range of 2 to 8 mg g fw $^{-1}$. As fruit developed beyond class 4, glucose, fructose, and sorbitol concentrations increased, while sucrose decreased to about half the original concentration. Glucose and fructose increased 10-fold in 'Smoky' and 'Northline' fruit during development. In mature fruit, sucrose concentrations were less than 1% of glucose or fructose concentrations, which were approximately equal at 3.6% each of total fresh weight. Strawberry and raspberry are similar to saskatoon in containing about equal amounts of glucose and fructose

(Makinen and Söderling 1980). These sugars increase steadily upon maturation in most fruits; however, unlike saskatoons the proportions often change (Whiting 1970) as for example, in apples and pears which typically contain up to three times more fructose than glucose (Berüter 1985).

Sorbitol increased 3- to 4- fold (to about 21 mg g fw $^{-1}$) in fruit of both cultivars (Fig. 2-7). Between classes 8 and 9 sorbitol concentration dropped 36% in 'Northline', but remained constant in 'Smoky'. Sorbitol is the primary photosynthetic product and is the major translocatable carbohydrate in many species of Rosaceae, including apple (Loescher 1987). Sorbitol may play similar roles in saskatoon. In apples, sorbitol is metabolized mainly to fructose and starch, and later during fruit growth, before the onset of the climacteric, starch hydrolysis begins (Berüter 1985). We could not detect starch in fruit at any stage and it is thus tempting to speculate that sorbitol is the precursor to fructose and glucose; however, in contrast to apple, saskatoon fruit does not transform glucose into starch. Stone fruits contain significant levels of sorbitol, and starch-to-sugar conversions are also not involved in ripening (Brady 1993). On average, total sugar concentration rose from 18 to 90 mg g fw $^{-1}$ as saskatoon fruit developed from class 1 to 9. Because the levels of sorbitol, glucose, and fructose increased significantly during ripening, there would be no quality advantage to harvesting fruit earlier than class 8 or 9.

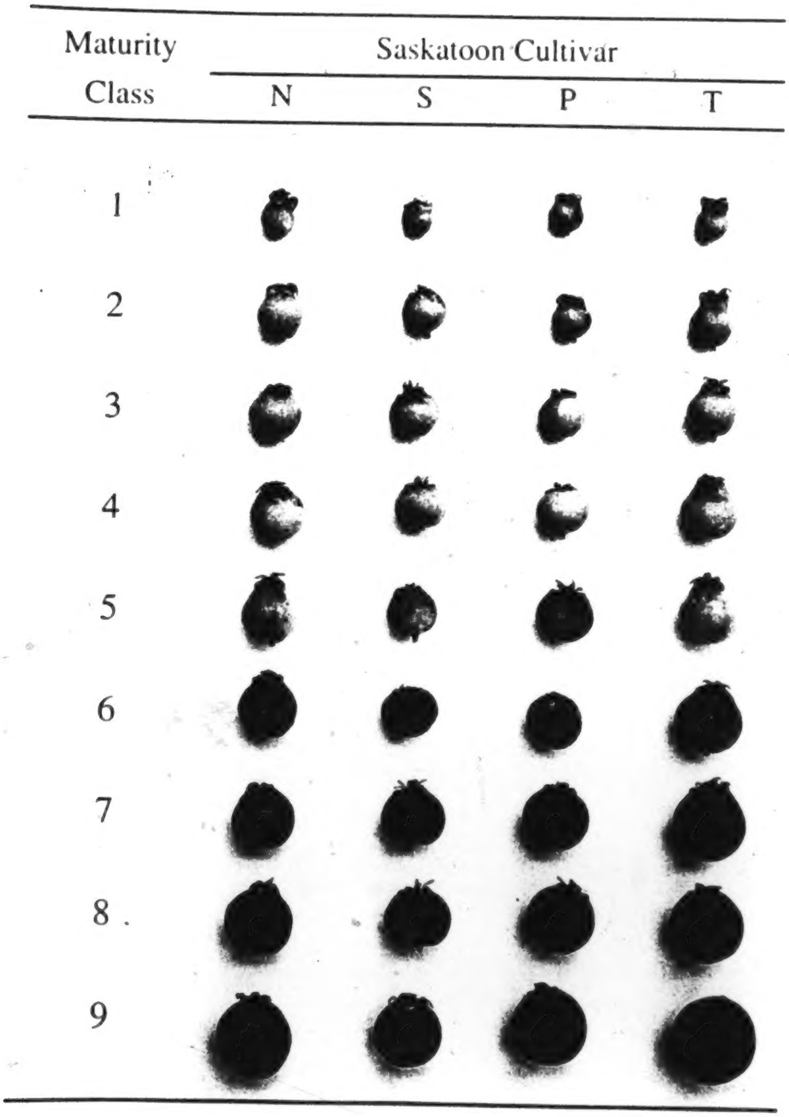
The organic acids in saskatoon fruit were primarily intermediates of the Krebs cycle (succinate, malate, citrate, cis-aconitate, pyruvate, fumarate), shikimic acid pathway (quinate), and glyoxylate pathway (oxalate). Galacturonate, a major component of pectin, was also identified. The profile of these acids shifted during development (Figs. 2-8 and 2-9), with succinate predominant in immature fruit and malate predominating in mature fruit. Succinate declined from an average of 16 mg g fw $^{-1}$ in class 1 fruit to 1 mg g fw $^{-1}$ in class 9 fruit. Malate concentration decreased marginally between classes 1 and 3, then increased 1.5-fold to class 6 in 'Smoky' fruit and 2.8-fold to class 5 in 'Northline' fruit, before declining to near original levels of 2.5 and 3.5 mg g fw $^{-1}$ in class 9 'Smoky' and 'Northline' fruit, respectively. Malate accounted for about 50% of the total organic acids (Fig. 2-10) in ripe fruit. Malate is the major acid in ripe apples (Ulrich 1970) and serves as a major respirable substrate as the fruit passes through the climacteric (Hulme and Rhodes 1971). Changes in succinate concentration with advancing maturity were greater than with any of the other acids, declining 14- to 18-fold during ontogeny of the fruit (Fig. 2-8). Between classes 5 and 9, however, malate and succinate concentrations declined at about the same rate. Trends in malate concentrations during development were very similar to trends in TA levels, indicating that malate may be dictating TA levels.

The minor organic acids identified in 'Smoky' and 'Northline' fruit included quinate, galacturonate, citrate, pyruvate, cis-aconitate, fumarate, and oxalate. Quinate concentrations were lower in 'Northline' than in 'Smoky' fruit and declined from class 1 to 3 in fruit from both cultivars (Fig. 2-8). Quinate was undetectable in fruit developing beyond class 3. Galacturonate levels ranged from 1.4 to 0.1 mg g fw^{-1} and, in general, declined throughout development (Fig. 2-8). In 'Smoky' fruit, citrate declined about 2-fold (to 200 µg g fw^{-1}) during development from class 1 to 9 (Fig. 2-9). The loss of citrate was even greater in 'Northline' fruit, falling 8-fold from class 1 to 5, after which it was no longer detectable. Pyruvate was detectable in 'Northline' fruit at all stages but only for classes 2 to 4 in 'Smoky' fruit. In 'Northline' fruit, pyruvate concentration was highest in class 5 (225 µg g fw^{-1}). Pyruvate was less than 40 µg g fw^{-1} when it was detected in 'Smoky' fruit. Changes in cis-aconitate concentrations during development were similar to those for malate with a decrease from classes 1 to 4, an increase to a maximum at class 6, and then a decline to near original levels at class 9. Relative to the other organic acids, fumarate was present in trace amounts and levels fell to less than 1 µg g fw^{-1} as fruits developed from classes 1 to 9. Oxalic acid was also detectable in fruit of maturity classes 1 to 4 for both cultivars, averaging 27 µg g fw^{-1} in 'Northline' and 20 µg g fw^{-1} in 'Smoky' fruit (data not shown). In total, organic acid concentrations decreased 5-fold for 'Smoky' fruit and 3-fold for 'Northline' fruit as they developed from classes 1 to 9 (Fig. 2-10). Organic acids account for 0.4 to 1.0 % of the fresh weight of ripe pome fruits (Hulme and Rhodes 1971), and saskatoons were consistent with this at 0.5 % (Fig. 2-10). Only three of these acids (malate, oxalate, and citrate) have previously been reported in saskatoon fruit (Wolfe and Wood 1971).

Nitrogen content (dry weight basis) increased slightly as fruit developed from classes 1 to 2, reaching 2.7 % in 'Northline' and 2.4 % in 'Smoky' fruit, then dropped 2.2-fold as fruit developed to class 9 (Fig. 2-11). Collectively, organic acids, carbohydrates, chlorophyll, anthocyanins, phenolics, and nitrogen accounted for 24.4% of the dry weight of a typical maturity class 1 'Smoky' fruit, and 52.2% of the dry weight of a typical maturity class 9 fruit. This maturity class 9 'Smoky' fruit had a pH of 4.4 and contained 21% dry matter, of which 1.1% was nitrogen. Furthermore, this fruit contained 16.4% soluble solids, of which 7.4% was reducing sugars, 2.0% was sorbitol, 0.03% was sucrose, 0.25% was malate, and 0.08% was succinate.

In summary, development of saskatoon fruit was associated with changes in flavour components such as sugars, organic acids, and phenolics, as well as with changes in firmness and color. Gross changes in color during fruit development were largely a consequence of chlorophyll degradation and synthesis of anthocyanins. Although environmental influences will contribute some variation to the physicochemical parameters characterized for each maturity class, trends in these parameters are not expected to deviate greatly from that reported here. The nine-level saskatoon fruit maturity index developed here is intended to be used as a quick reference system by other researchers studying fruit physiology, biochemistry, pathology, etc., who wish to identify and refer to a particular developmental stage, and will thus reduce or eliminate the need for an exhaustive description during each communication. This index has already been used successfully by other researchers studying the effects of growth regulators on fruit development (McGarry 1996). We also recommend that the saskatoon industry recognize and adopt this system. Using this index, a grower may easily recognize specific stages where susceptibility to disease or insect damage is high, thus justifying pesticide application. Alternatively, it may be used to improve scheduling of irrigation and fertilizer applications, potentially contributing to the improvement of production practices.

Fig. 2-1. Maturity class index for saskatoon fruit cvs. Northline (N), Smoky (S), Pembina (P) and Thiessen (T). Fruit in each of the nine maturity classes were sorted subjectively on the basis of size and color, both of which are quantified for cvs. Northline and Smoky in Figures 2-3 and 2-4.


Maturity Class
Saskatoon Cultivar
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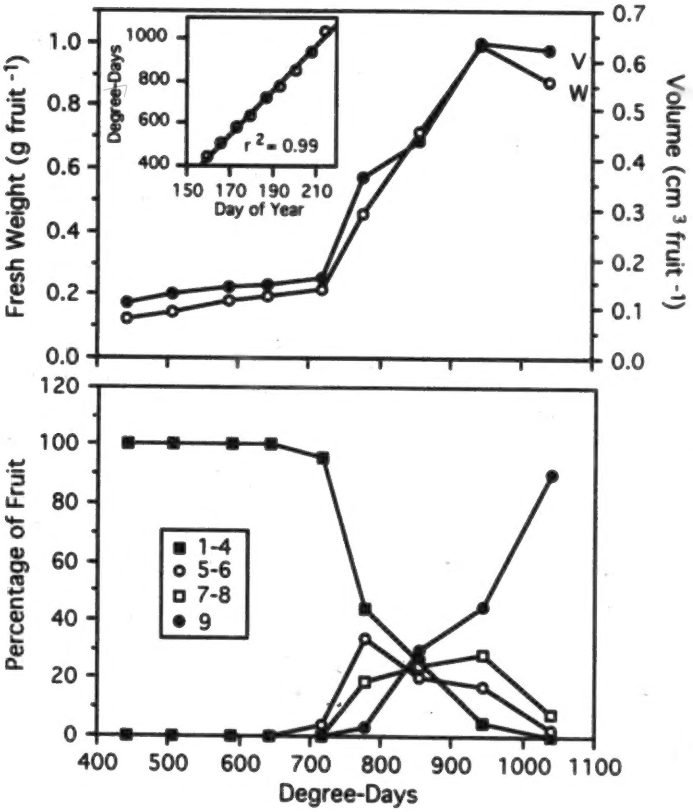


Fig. 2-2. Growth in fresh weight (W) and volume (V) of cv. Smoky fruit and percentage distribution of fruit by number among the maturity classes (see Fig. 2-1) with increasing degree days during the 1993 growing season. Inset shows the trend in degree days with time of harvest. While percentage of fruit in each of the nine maturity classes was determined at each harvest, data from selected maturity classes were combined for graphical clarity. Weight and volume data are means of 50 fruit ± standard errors (which are eclipsed by the symbols).

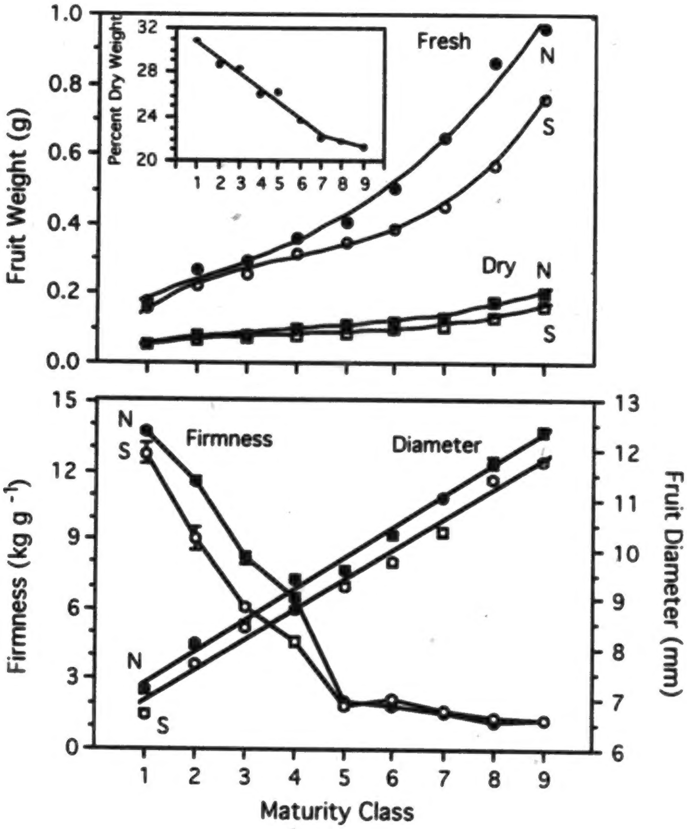


Fig. 2-3. Changes in fruit fresh weight, dry weight, firmness and diameter with advancing stages of maturity (see Fig. 2-1) for the cvs. Northline (N) and Smoky (S). Fresh weight of 'Northline' fruit = 1.41E-1 + 5.39E-2 MC - 3.93E-3 MC^2 + 9.46E-4 MC^3, where MC = maturity class, $R^2 = 0.99$. Fresh weight of 'Smoky' fruit = 2.97E-2 + 1.45E-1 MC - 2.86E-2 MC^2 + 2.39E-3 MC^3, $R^2 = 0.99$. Dry weight of 'Northline fruit = 2.77E-2 + 3.51E-2 MC - 6.25E-3 MC^2 + 5.13E-4 MC^3 $R^2 = 0.99$. Dry weight of 'Smoky' fruit = 2.65E-2 + 2.85E-2 MC - 5.62E-3 MC^2 + 4.61E-4 MC^3, $R^2 = 0.99$. Diameter of 'Northline ' fruit = 6.65 + 0.63 MC, $r^2 = 0.99$. Diameter of Smoky' fruit = 6.33 + 0.60 MC, $r^2 = 0.99$. Inset shows trend in fruit dry weight, expressed as a percentage of fresh weight, with advancing maturity class. Fruit weight data are means of 50 fruit ± standard error. Firmness and diameter data are means of 3 replicate, 15 g samples and 50 fruit ± standard errors, respectively.

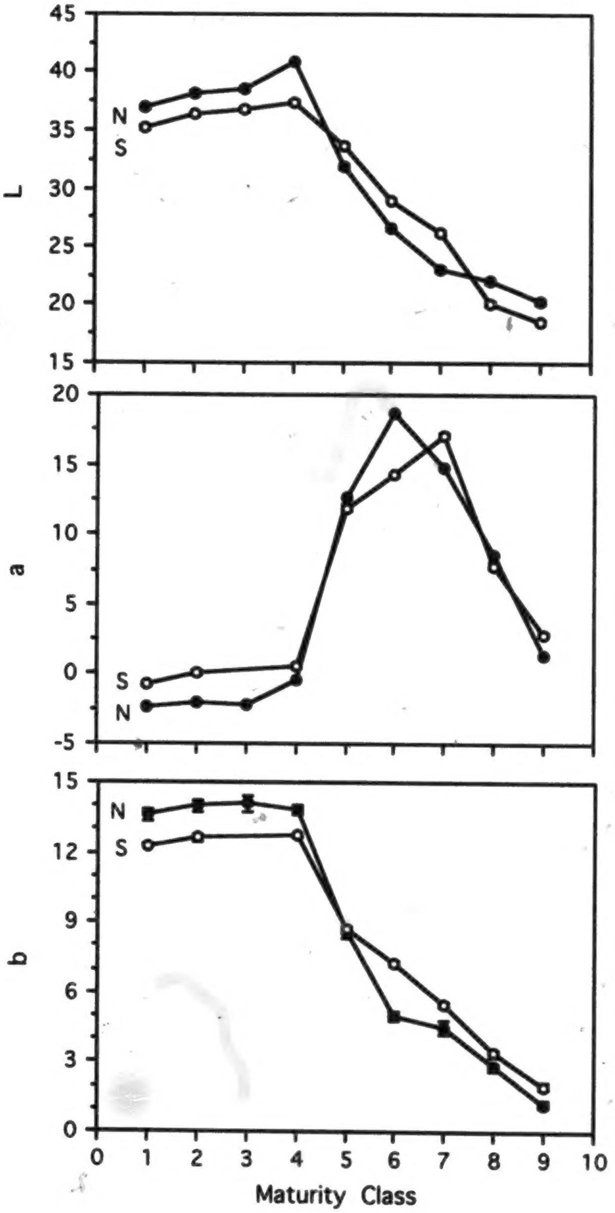


Fig. 2-4. Changes in Hunterlab colorimeter 'L', 'a' and 'b' values for cvs. Smoky (S) and Northline (N) fruit with advancing maturity class (see Fig. 2-1). Data are means ± standard error of 3 replicate, 20 g samples of fruit.

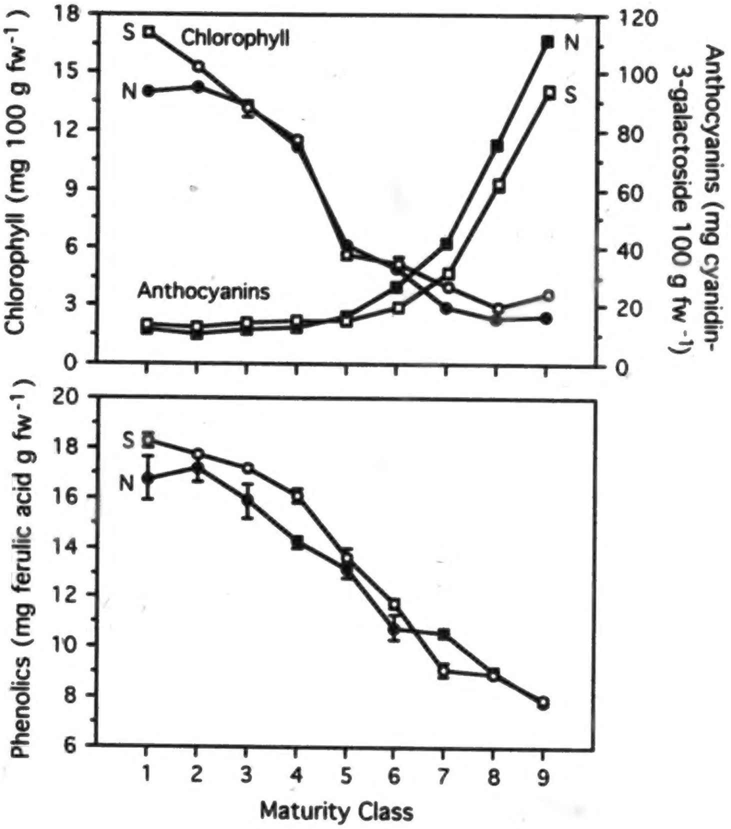


Fig. 2-5. Changes in chlorophyll, anthocyanins and total phenolic titres of cvs. Northline (N) and Smoky (S) fruit with advancing maturity class (see Fig. 2-1). Total anthocyanins and phenolics are expressed as milligram cyanidin-3-galactoside and ferulic acid equivalents, respectively. Data are means ± standard error of 3 replicate samples of fruit.

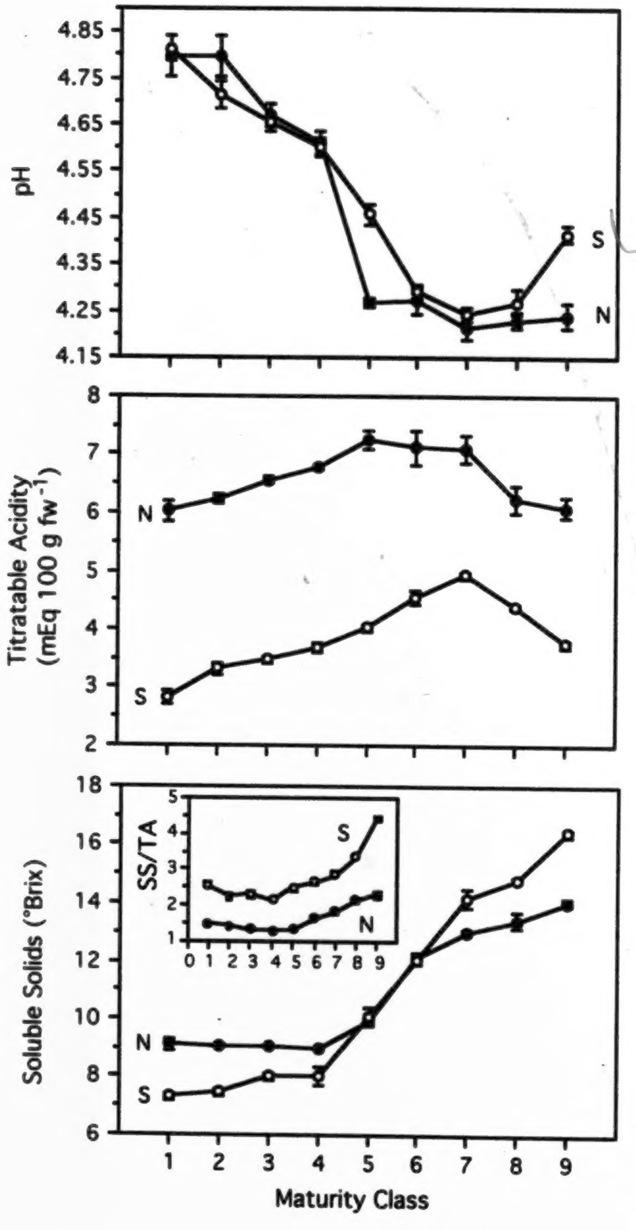


Fig. 2-6. Changes in pH, titratable acidity, soluble solids and soluble solids/titratable acidity ratio (SS/TA) in cvs. Northline (N) and Smoky (S) fruit with advancing maturity class (see Fig. 2-1). Data are means ± standard error of 3 replicate samples of fruit.

Fig. 2-7. Changes in glucose, fructose, sorbitol and sucrose concentrations in cvs. Northline (N) and Smoky (S) with advancing maturity class. Data are means ± standard error of 3 replicate, 5 g samples of fruit.

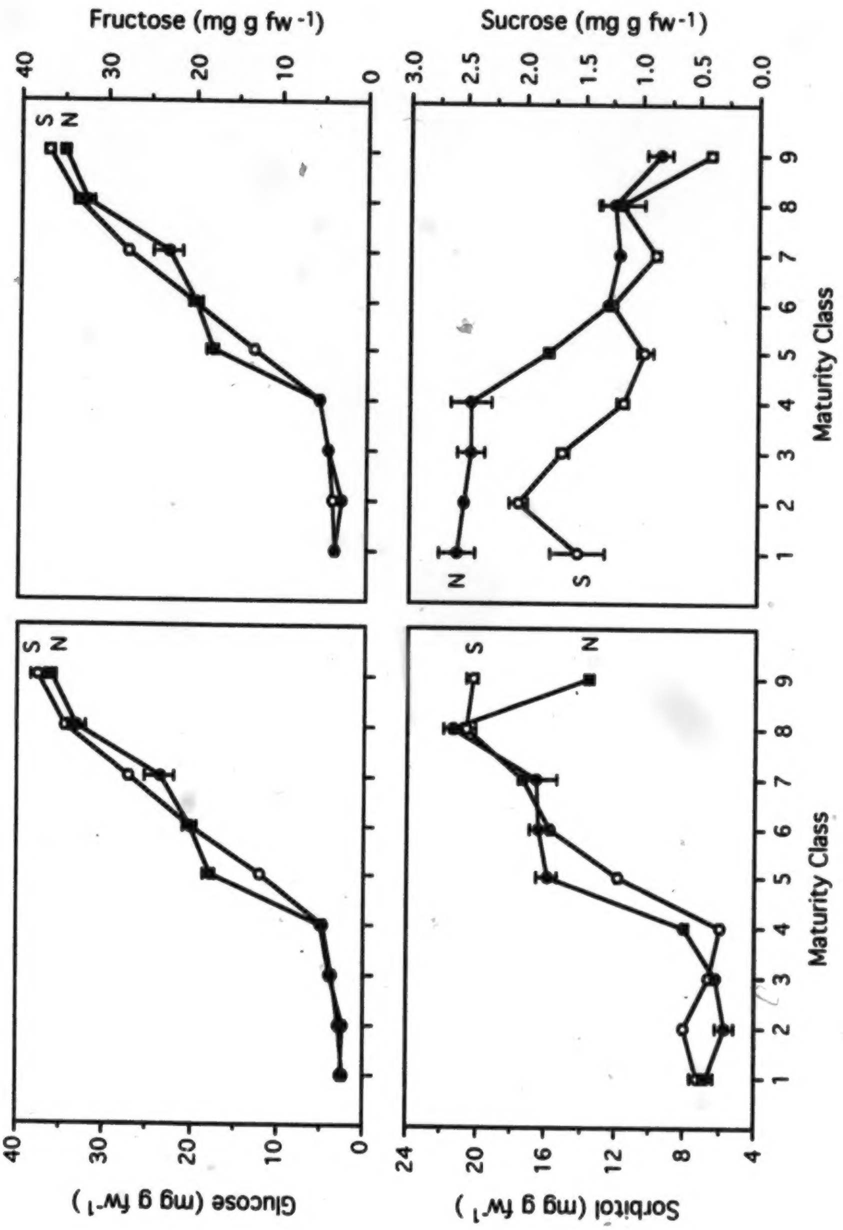

Glucose (mg g fw-1)
Fructose (mg g fw -1)
Sorbitol (mg g fw-1)
Sucrose (mg g fw-1)
Maturity Class
Maturity Class
S
N
40
30
20
10
0
24
20
16
12
8
4
3.0
2.5
2.0
1.5
1.0
0.5
0.0
1
2
3
4
5
6
7
8
9

Fig. 2-8. Effect of fruit maturity class on concentrations of major (succinate and malate) and minor (quinate and galacturonate) organic acids. Data are means ± standard error of 3 replicate, 5 g samples of fruit.

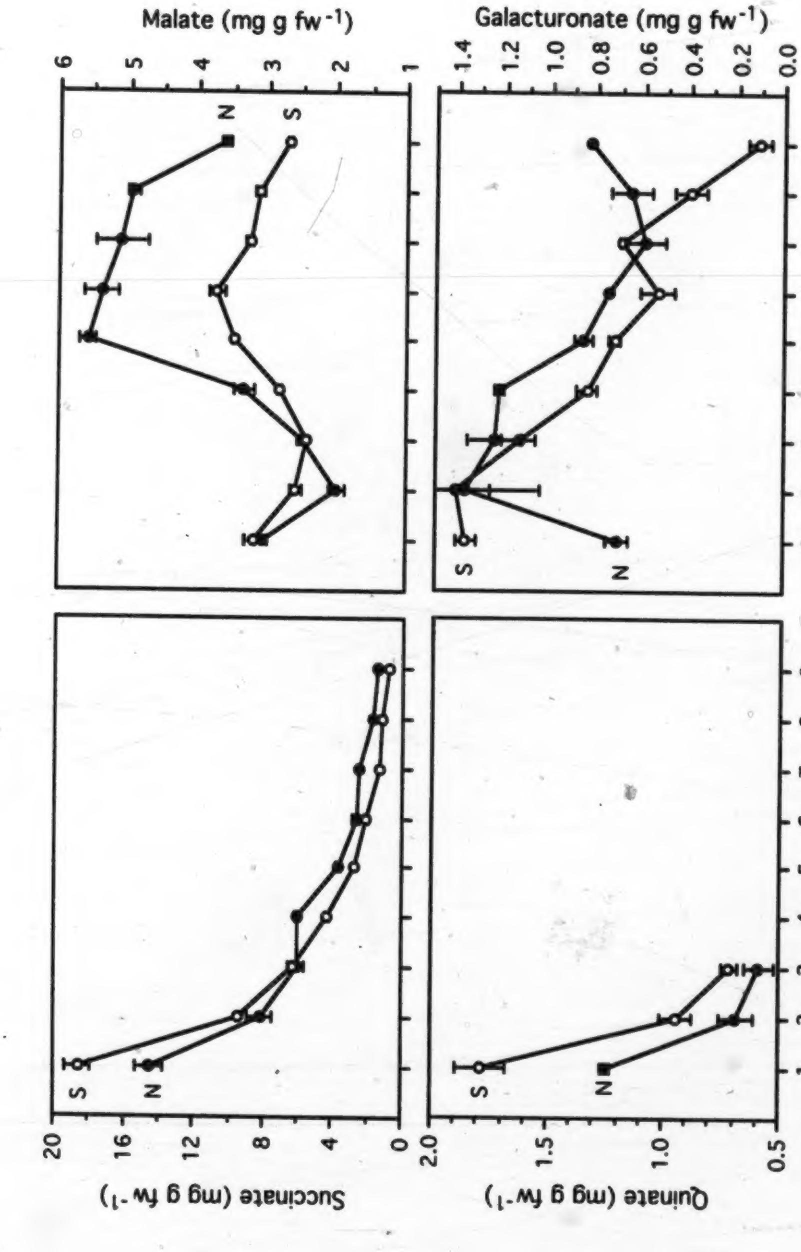
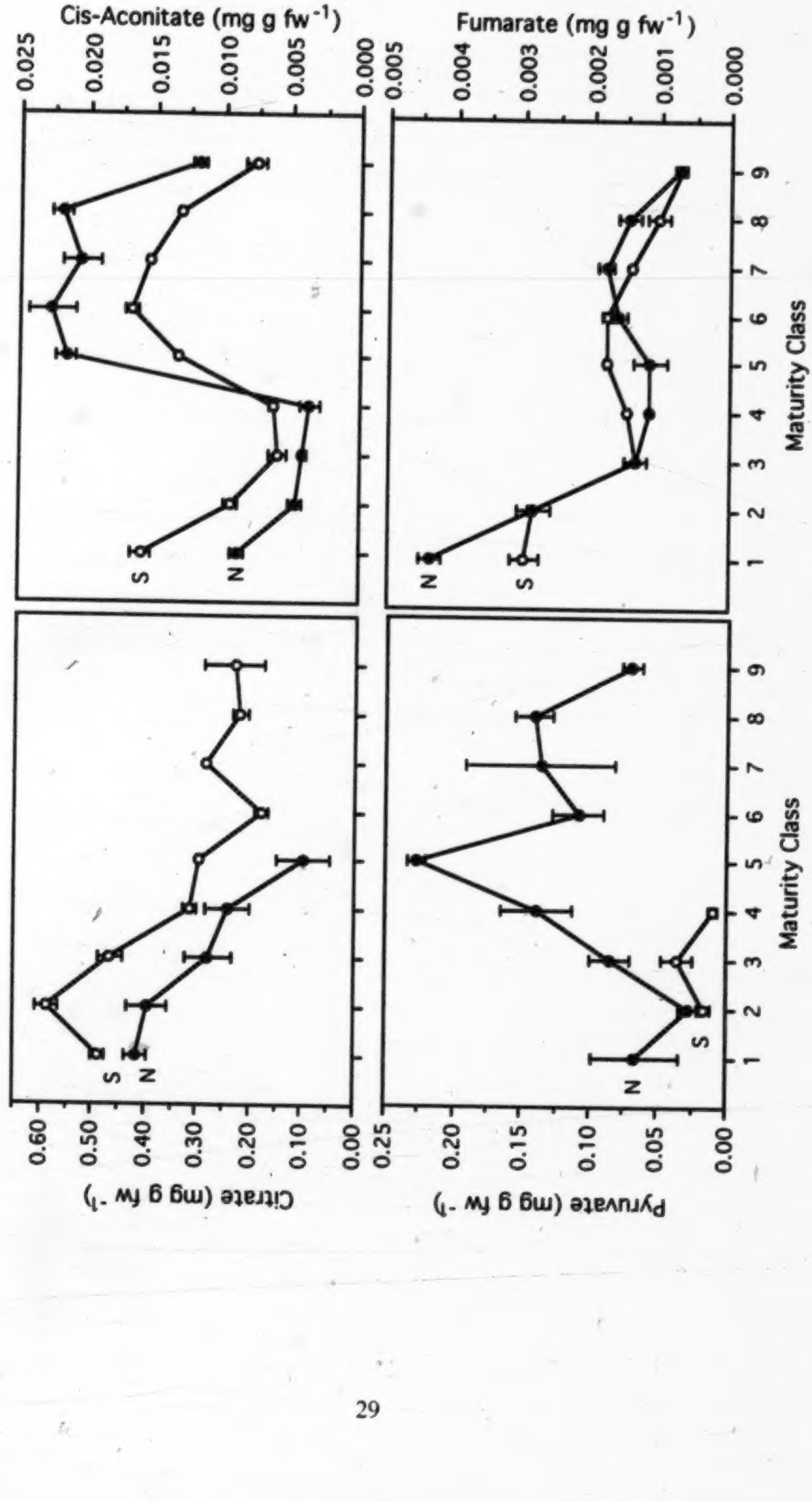

Succinate (mg g fw-1)
20
16
12
8
4
0
Quinate (mg g fw-1)
2.0
1.5
1.0
0.5
Malate (mg g fw-1)
6
5
4
3
2
1
Galacturonate (mg g fw-1)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
S
N
Maturity Class
1
2
3
4
5
6
7
8
9

Fig. 2-9. Changes in concentrations of the minor organic acids citrate, cis-aconitate, pyruvate and fumarate in fruit with advancing maturity class. Data are means ± standard error of 3 replicate, 5 g samples of fruit.


Citrate (mg g fw -1)
0.60
0.50
0.40
0.30
0.20
0.10
0.00
Cis-Aconitate (mg g fw-1)
0.025
0.020
0.015
0.010
0.005
0.000
Pyruvate (mg g fw -1)
0.25
0.20
0.15
0.10
0.05
0.00
Fumarate (mg g fw-1)
0.005
0.004
0.003
0.002
0.001
0.000
S
N
Maturity Class
1
2
3
4
5
6
7
8
9

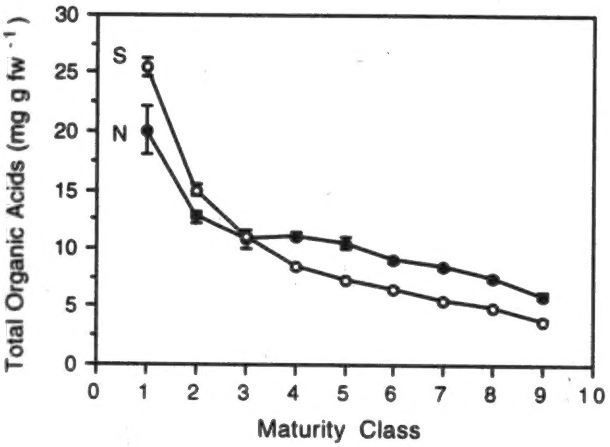


Fig. 2-10. Changes in total organic acid concentrations in cvs. Northline (N) and Smoky (S) fruit with advancing maturity class (see Fig. 2-1). Data are means ± standard error of 3 replicate, 5 g samples of fruit.

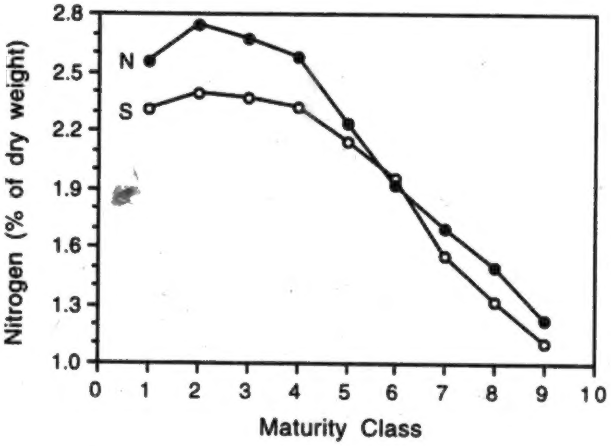


Fig. 2-11. Changes in total nitrogen concentrations in cvs. Northline (N) and Smoky (S) fruit with advancing maturity class (see Fig. 2-1). Data are means ± standard error (eclipsed by symbols) of 2 replicates.

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Chapter III

Regulation of Ethylene Production and Ripening By Saskatoon (*Amelanchier alnifolia* Nutt.) Fruit

Abstract

Evidence for climacteric-type behavior was examined during maturation and ripening of saskatoon (*Amelanchier alnifolia* Nutt.) fruit. On a per fruit basis, respiration and ethylene production increased 78% and 400%, respectively, as fruit ripened on the plant and trends were consistent with those for climacteric-type fruits. When estimated on a fresh and dry weight basis, increased rates of ethylene production were still apparent during ripening, however respiration rate declined. Trends in respiration rates and endogenous ethylene levels of harvested fruit of nine maturity classes, from immature green (class one) to fully ripe and purple (class nine), were consistent with those of fruit growing on the plant. Tissue prints showed that ACC (1-aminocyclopropane-1-carboxylic acid) oxidase was distributed throughout the pericarp of fruit at all nine maturity stages and the enzyme was most concentrated in the immature stages on a per fruit basis. On a protein basis, ACC oxidase increased progressively with development of cv. Smoky fruit, but remained relatively constant over the nine maturity classes of cv. Northline fruit. In contrast, ACC oxidase levels were relatively low in cv. Pembina fruit over the first four maturity classes, increased substantially as fruits developed from class four to five, then remained constant as fruit ripened to maturity class nine. Infiltrating harvested 'Pembina' fruit of maturities one to nine with 1 mM S-adenosylmethionine had no effect on the rate of ethylene production; however, when fruit were infiltrated with 1 mM ACC, ethylene production by immature fruit (maturity classes one to three) increased 28- to 108-fold, compared with an average of only 7-fold for fruit of maturity classes four through nine. Preharvest treatment of class three fruit with ACC, via a cotton thread through the base of the infructescence, induced ripening to maturity class eight within 5 days, while fruit on untreated infructescences required 15 days to reach class eight. Vacuum infiltrating class four fruit with the ACC synthase inhibitors, aminooxyacetic acid (AOA) and aminoethoxyvinyl glycine (AVG), inhibited ethylene production and color development substantially. The inhibiting effect of AOA on ripening was eliminated when fruit were infiltrated with equimolar concentrations of AOA and ACC. Cobalt, an ACC oxidase inhibitor, also inhibited ethylene production and ripening. Collectively, our

results indicate that ethylene synthesis by preclimacteric fruit is limited by the availability of ACC, ethylene is responsible for initiating ripening, and saskatoon fruit are climacteric.

Introduction

The saskatoon (*Amelanchier alnifolia* Nutt.) is a fruit-bearing shrub of the Rosaceae that is native to the Canadian Prairies and Northwestern United States (Harris, 1972). These miniature pomes are 1 to 1.5 cm in diameter, held in racemelike, determinate inflorescences (Steeves and Steeves, 1990), and are purple at maturity. The fruit is consumed fresh or is machine harvested and processed. While expansion of the saskatoon industry has spurred increased research on fruit growth and development (McGarry, 1996), epidemiology (Pluim et al., 1994), fruit chemical composition and oxidative metabolism (Rogiers and Knowles, 1997; Rogiers et al., 1997), little is known about the fruits' ripening physiology and postharvest behavior. Characterizing the metabolic bases of ripening and subsequent deterioration is a prerequisite to developing handling, transport and storage systems that will maintain fruit quality after harvest.

The objectives of this study were to determine if ethylene is involved in saskatoon fruit ripening and to classify the fruit as climacteric or nonclimacteric based on changes in respiration and ethylene production rates during ripening. Accordingly, trends in respiration and ethylene production were characterized throughout development of fruit on the plant. Internal ethylene content, ACC oxidase levels and respiration rates were also compared among nine maturity classes of several cultivars of fruit immediately after harvest. Ripening and endogenous ethylene production responses to treatment with ACC and ethylene biosynthesis inhibitors indicated a central role for ethylene in fruit ripening. Our results show that saskatoon fruit are climacteric.

Materials and Methods

Preharvest respiration and ethylene production during fruit development

Three-year-old potted saskatoon (*Amelanchier alnifolia* Nutt. cv. Northline) shrubs, obtained from the Crop Diversification Centre North (Edmonton, Alberta) in May, were acclimated for 1 week to room temperature under natural light supplemented with high pressure sodium lamps (Sylvania 400 W, 250 $\mu E\ m^{-2}\ s^{-1}$ measured at top of canopy, 16 h photoperiod). Temperature fluctuated between 22°C (night) and 26°C (day) and plants were watered as needed. At the end of the acclimation period, a 3 cm long by 1.5 cm wide (7 mL) glass tube, sealed on one end with a rubber septum, was placed over individual fruit (Fig. 3-1) and an inert putty (Fun

Tac™, LePage, Brampton, Ontario) was used to seal around the peduncle, enclosing the fruit inside the chamber. Only immature green fruit of maturity classes two to three (Rogiers and Knowles, 1997) were selected for these studies. Inlet and outlet hypodermic syringe needles (25 gauge) were inserted into the rubber septum and a constant air flow (21% O_2, 0.0015% CO_2, balance N_2) of 2 mL min^{-1} was established through each chamber.

The outflow from each chamber (four chambers in all) was directed through an LI-6262 CO_2/H_2O infra-red gas analyzer (LI-COR, Inc., Lincoln, Nebraska) by a three-way solenoid valve for 45 min, whereupon the CO_2 concentration was recorded and a computer switched to the next valve, directing the outflow from the next fruit through the analyzer. Carbon dioxide concentration was thus recorded every 3 h for each fruit and respiration was calculated and expressed as μL CO_2 per fruit per hour. Gas samples (1 mL) were also taken from the outlets for ethylene analysis, approximately every 12 to 24 h. Ethylene was analyzed on a Photovac 10A10 GC equipped with a photoionization detector and an 8 ft. by 1/8 inch teflon column packed with Porasil B (100-150 mesh). The carrier gas (air) flow rate was 15 mL min^{-1} and the column was maintained at 23°C. Respiration and ethylene production by each fruit were monitored at least until fruit had fully developed to maturity class nine (purple stage, see Rogiers and Knowles (1997)). This entailed about two weeks of continuous monitoring for each fruit.

Respiration and ethylene production rates were also compared on a fresh and dry weight basis. Fruit weights in the chambers were derived by first estimating fruit diameter (mm) at any particular time (diameter = $9.0678 - 2.7455e^{-1}(day) + 1.1275e^{-1}(day)^2 - 4.5325e^{-3}(day)^3$, $r^2 = 0.99$, $P<0.01$), then calculating fruit weights (g) based on the linear relationships with diameter (mm) (diameter = 7.3862 + 5.0179(f. wt.), $r^2 = 0.93$, $P<0.01$; diameter = 6.59498 + 30.205(d. wt.), $r^2 = 0.90$, $P<0.01$). Diameter-time and weight-diameter algorithms were produced for cv. Northline fruit from the same plants and under the same growing conditions (see above) as those in the preharvest fruit respiration and ethylene study. Changes in respiration and ethylene production with development were characterized for three cultivars, three plants per cultivar and at least four fruits per plant. Since changes with development were similar among cultivars, only data for cv. Northline are presented.

Postharvest respiration and fruit ethylene content

Saskatoon fruit, cvs. Thiessen, Smoky, Pembina (*Amelanchier alnifolia* Nutt.) and Parkhill (*Amelanchier sanguinea* (Pursh.) DC.), were hand harvested from 5 to 8 year old plants in a commercial orchard at Spruce Grove, Alberta. The fruit were sorted into nine maturity classes based on size and color using an index system developed specifically for saskatoon fruit (Rogiers and Knowles, 1997). Fruit of maturity classes one to four were relatively immature, firm and green and contained high concentrations of chlorophyll. Chlorophyll content declined from stage four to nine, with a concomitant increase in anthocyanins. Fruit of maturity class five were 50% pinkish-red, six were light red, seven were dark red, eight were dark red with purple hues, and nine were purple. Fruit diameter increased linearly (r^2=0.99, P<0.01) at an approximate rate of 0.6 mm per maturity class starting from an average of 7 mm for maturity class one (see Rogiers and Knowles, 1997).

To determine respiration rates, fruit of each cultivar and maturity class were placed individually inside 3 cm long by 1.5 cm wide (7 mL) glass tubes. The tubes were sealed at both ends with rubber septa and inlet and outlet ports were established by placing 18 gauge hypodermic needles through each septum. A constant (15 mL min^{-1}), humidified airflow (21% O_2, 0.0015% CO_2, balance N_2) was established through each chamber. Following acclimation of fruit in the chambers at 23°C for 2 h, 1 mL gas samples were collected from the outlets and analyzed for CO_2 with a Hewlett-Packard 5890A gas chromatograph. The GC was equipped with a 2.4 m stainless steel column (3.2 mm o.d.) packed with HayeSep T (Hewlett Packard) and a thermal conductivity detector. The carrier gas (He) flow rate was 30 mL min^{-1} and the column was isothermal at 100°C. Injection and detector port temperatures were 140°C. Respiration rates were determined for three replicates of each of nine maturity classes of fruit from four cultivars and are expressed as μL CO_2 h^{-1} on a fresh weight and whole-fruit basis.

Endogenous ethylene was extracted from fruit of maturity classes one to nine by modifying the methods of Beyer and Morgan (1970). Approximately 4 L of H_2O was placed in a 6 L desiccator and degassed under vacuum (740 mm Hg) for 8 h. The stem of a 200 mL glass funnel was closed with a rubber septum and the funnel was inverted and submerged in the H_2O. The degassed H_2O was then displaced into the funnel by evacuating air through the septum with a 50 mL syringe. To extract gas from the fruit, 25 g of fruit were immersed in 0.01% Tween 20 for 15 sec and placed within the cone of the funnel, taking care not to introduce any air. The desiccator was closed, evacuated to 17 mm Hg, and gas from the fruit was collected in the stem of the funnel for 2 min. The vacuum was then released and 1 mL of gas was withdrawn through the septum and analyzed for ethylene by GC, as described above.

Western analysis

Enzyme extracts were prepared from the pericarp of fresh (cv. Pembina) and frozen (cvs. Northline and Smoky) fruit of maturity classes one to nine. Fruit (5 g fresh weight) was extracted in 5 mL of potassium phosphate buffer (0.1 M, pH 7.0) containing 0.05% (w/v) Triton X-100, 1 mM each of PMSF and EDTA, 5 mM DTT and 5% (w/v) PVPP by gentle grinding (mortar and pestle) to avoid maceration of the seeds. The homogenate was filtered through Miracloth and was centrifuged at 30,000 g for 15 min. The supernatant was desalted through a PD-10 Sephadex G-25M column (Sigma). Protein was determined by the BCA assay (Stoscheck, 1990) using protease-free BSA as a standard. Samples were diluted 1:1 with SDS-buffer [62.5 mM Tris (pH 6.8) containing 4.9% (v/v) glycerol, 0.97% (w/v) SDS, 4.9% (v/v) ß-mercaptoethanol and 0.002% (w/v) bromophenol blue] and incubated at 95°C for 10 min. Proteins (80 µg) were then separated by discontinuous SDS-PAGE on a 12% separating (4% stacking) gel (Laemmli, 1970). Proteins in the gels were electroblotted (100 V for 1 h) to nitrocellulose and probed with polyclonal anti-apple ACC oxidase (Dilley et al., 1995) diluted 1:8000 with blocking buffer. Immunoblots were developed with anti-rabbit alkaline phosphatase-conjugated secondary antibody (see Kumar and Knowles, 1996). An extract of apple (cv. Spartan) pericarp was also prepared (as above) and proteins (5 µg) were electrophoresed and immunoblotted alongside saskatoon samples for comparison.

Tissue printing

Freshly harvested cv. Pembina fruit of maturity classes one to nine were halved transversely and placed cutside down on nitrocellulose paper. The fruit halves were removed after 1 min and the prints were air-dried at room temperature. Tissue prints were blocked for 1 h with phosphate buffer saline [10 mM NaH_2PO_4 buffer (pH 7.2) containing 150 mM NaCl, 0.3% (w/v) Tween 20] which also removed anthocyanins and other fruit phenolics from the nitrocellulose. The prints were then incubated for 2 h with antibody against ACC oxidase, diluted 1:8000 with blocking buffer. Prints were developed with alkaline phosphatase-conjugated secondary antibody as above.

Induction and Inhibition of Ethylene Biosynthesis

Fruit (cv. Pembina) were harvested from potted 3-year-old plants, sorted into nine maturity classes as previously described, weighed and placed individually in 15 mL test tubes. Within 5 min from harvest, four replicates of fruit of each maturity class were vacuum infiltrated (580 mm Hg, 1 min) with the appropriate precursor or inhibitor of ethylene synthesis (see below, all chemicals from Sigma). The fruit were then transferred to clean test tubes which were sealed with rubber septa. After 3 h of incubation at 23°C, a 1 mL sample of headspace gas was removed and analyzed for ethylene by GC, as described above. Preliminary studies showed that ethylene accumulation was linear through 3 h of incubation under these conditions.

To determine the effects of developmental stage of fruit on ability to produce ethylene, fruit of maturity classes four, seven and nine were infiltrated with 0, 0.1, 0.25, 0.5, 1.0, 5.0 and 10 mM ACC in MES buffer (50 mM, pH 7.0). The fruit were incubated at 23°C for 3 h and ethylene production was assessed (as above). The capacity for ethylene production was further investigated by infiltrating fruit of maturity classes one through nine with one of three treatment solutions: a control consisting of MES buffer (50 mM, pH 7.0), 1 mM SAM, or 1 mM ACC. Both ethylene precursors were made up in MES buffer (50 mM, pH 7.0) and ethylene production was assessed as outlined above.

The ability to inhibit ethylene biosynthesis and slow ripening was also evaluated using AOA, a potent ACC synthase inhibitor (Yang and Hoffman, 1984). In this study, maturity class four fruit were infiltrated with either 50 mM MES buffer (pH 7.0), 5 mM AOA, 1 mM ACC, or a solution containing 5 mM AOA and 1 mM ACC (all in MES buffer, 50 mM, pH 7.0). Treated fruit were placed individually in 15 mL test tubes which were left unsealed, but were covered with moistened chromatography paper to maintain humidity and prevent desiccation. The fruit were incubated at 23°C for 0.5, 1.5 and 3.5 days. Three hours prior to the end of each incubation interval, tubes were closed with septa, incubated for 3 h at 23°C, and headspace gas samples were analyzed for ethylene. Fruit were photographed after 3.5 d of ripening to document effects on fruit color development. In another study, maturity class four fruit were infiltrated with MES buffer (50 mM, pH 7.0), 1 mM AVG (ACC synthase inhibitor) in MES buffer, or 7 mM $CoCl_2$ (ACC oxidase inhibitor) in MES buffer, and ethylene production rate was assessed 1 day after treatment, as described above.

Preharvest effects of ACC on fruit development

Potted shrubs (cv. Pembina), bearing fruit of average maturity class three, were held under the lighting and temperature regimes described for the preharvest respiration and ethylene production studies. Infructescences containing eight to twelve fruit were selected for evaluation of the effects of ACC on development. Approximately 1 cm of a 7 cm long cotton thread was pulled through the base of the main peduncle of an infructescence. The long end of the thread was pulled through a 5 cm long capillary tube (1.2 mm i.d.) which was inserted through a hole in the cap of a light-tight test-tube (6.5 mL). The tubes contained 6.0 mL of either 50 mM MES buffer, pH 7.0 (control) or MES buffer containing 1 mM ACC (five replicates of each treatment). The tip of the capillary tube was placed against the peduncle of the infructescence to prevent desiccation of this end of the thread. The 1 cm of thread on the other side of the peduncle remained exposed so that evaporation would pull the solution through the base of the infructescence. Solution uptake was verified by loss from the tubes over the 5 day treatment interval. Maturity class of each fruit within an infructescence was recorded daily for 5 days, using the saskatoon maturity index system (Rogiers and Knowles, 1997). The infructescences were then harvested, photographed, each fruit was weighed, and anthocyanin concentration was determined collectively for the fruit from each infructescence according to Rogiers and Knowles (1997), as modified from the pH differential method of Fuleki and Francis (1968).

Results

Respiration and ethylene production of 'Northline' fruit were monitored continually during development on the plant. The respiration rate of immature green fruit (maturity class two) was about 70 μL CO_2 fruit^{-1} h^{-1} and declined to a low of 50 μL CO_2 fruit^{-1} h^{-1} during the first 6 days of development (Fig. 3-2). By day 7, fruit had developed to maturity class four (greenish-yellow), as defined by Rogiers and Knowles (1997). Respiration increased 50% (to 75 μL CO_2 fruit^{-1} h^{-1}) from day 6 to 10 before declining over the remaining 3 days of study. Fruit maturity had advanced from class six (light red) at day 8 to class nine (fully ripe and purple) by day 9. Respiration rate thus increased substantially during ripening, with a maximum occurring about 1 day after the attainment of full ripeness. On a weight basis, respiration rate declined rapidly as fruit developed from maturity class two to four (0 to 7 days), increased slightly to maturity nine (at day 9), then fell gradually through the remaining 3 days.

The rate of ethylene production remained stable over the first six days at about 0.4 nL fruit^{-1} h^{-1} (Fig. 3-2). It then increased as fruit matured to class four, concurrent with the increase in respiration rate. Fruit ethylene production increased about 6-fold from day 6, reaching 2.5 nL fruit^{-1} h^{-1} with full maturity at day 9. Similar trends in ethylene evolution were evident on a fresh and dry weight basis. While the timing of ripening varied among fruit, those fruit that developed completely to maturity nine had similar trends in respiration and ethylene production rates. In contrast, one particular fruit that did not develop past class three over 16 days of monitoring, respired at a constant 50 μL CO_2 fruit^{-1} h^{-1} after an initial decline (data not shown). The rate of ethylene production also remained constant for this fruit at less than 1 nL fruit^{-1} h^{-1}, thus indicating that the ethylene and respiration increases characterized for cv. Northline fruit in Fig. 3-2 were associated with ripening. Similar trends in respiration and ethylene production rates during ripening were also found for cvs. Pembina and Smoky fruit (data not shown).

Trends in postharvest ethylene content and respiration rates of nine maturity classes of fruit from four cultivars (Fig. 3-3) were consistent with those characterized in the preharvest study

(Fig. 3-2). Endogenous ethylene concentration rose 2- to 4-fold as fruit developed from maturity class one (immature green) to maturity class nine (purple) (Fig. 3-3). The increases, which became apparent as fruit matured from class three to four, extended to classes six through eight, depending on cultivar. Ethylene concentrations then either declined slightly as fruit developed to class nine ('Parkhill' and 'Pembina'), remained constant ('Thiessen'), or increased further ('Smoky'). 'Pembina' and 'Smoky' fruit accumulated 75 to 170% more ethylene during ripening than 'Thiessen' and 'Parkhill'. On a per fruit basis, respiration rates increased 2- to 3-fold during development and the increases were either concurrent with ('Smoky', 'Thiessen'), or lagged behind the rising ethylene concentrations ('Parkhill', 'Pembina'). Respiration rates on a fresh weight basis declined to less than half the original values as fruit matured from class one to class nine (Fig. 3-3, insets). Moreover, cvs. Pembina and Smoky had much higher respiration rates on a per g basis than Thiessen and Parkhill.

SDS-PAGE of proteins extracted from pericarp tissue showed that polypeptide patterns were similar through the first six maturity classes of cv. Smoky fruit (Fig. 3-4). Over this range of development, soluble protein was dominated by three major polypeptides at approximately 22, 39 and 45 kD. All three proteins increased in concentration (protein basis) as fruit developed from class one to three. Concentrations of the 39 and 45 kD proteins then remained constant through maturity six, while that of the 22 kD protein declined. Levels of these three proteins were substantially lower in fruit of maturity classes seven, eight and nine.

Tissue prints indicated that ACC oxidase was present in the pericarp of cvs. Pembina (Fig. 3-4), Northline, Smoky and Thiessen (prints not shown) at all nine developmental stages. ACC oxidase (35 kD) was most concentrated on a per fruit basis in immature class one fruit of all cultivars and declined with advancing maturity. Western analysis showed that saskatoon ACC oxidase cross-reacted with anti-apple ACC oxidase; however, apple (cv. Spartan) likely contained higher levels of the enzyme (compare immunoreactions for 5 μg and 80 μg protein loads for apple and saskatoon fruit, respectively). The enzyme increased on a protein basis with advancing maturity of cvs. Smoky and Pembina fruit. In cv. Pembina fruit, the largest increase in ACC oxidase occurred as fruit matured from class four to five, whereupon the level remained relatively constant through further development. In contrast, ACC oxidase increased progressively in Smoky fruit during development from maturity class four to nine. For both 'Pembina' and 'Smoky' fruit, the initial maturity classes over which ACC oxidase increased (classes four to five, Fig. 3-4) coincided with those at which ethylene content began to increase (Fig. 3-3). This indicates that ethylene may induce synthesis of ACC oxidase in select cultivars of saskatoon fruit, as has been shown in banana (Inaba and Nakamura, 1986) and other fruit and vegetative tissues (Gupta and Anderson, 1989; Schierle et al., 1989; Ievinsh et al., 1990). ACC oxidase levels in cv. Northline fruit were relatively high and constant throughout development (Fig. 3-4).

To determine whether ethylene biosynthesis is substrate limited, fruit of maturity classes four, seven and nine (cv. Pembina) were infiltrated with increasing concentrations of ACC and ethylene production was quantified. Averaged over maturity classes, ethylene production by fruit increased linearly with increasing ACC concentration (nL C_2H_4 g f wt^{-1} h^{-1} = 5.337 + 37.56[ACC], r^2=0.99, P<0.01) (Fig. 3-5A). The efficacy of ACC in stimulating ethylene production rate declined by 3.5 nL C_2H_4 g fwt^{-1} h^{-1} per mM ACC as fruit advanced in maturity from classes four to nine (Fig. 3-5A inset). This decreasing sensitivity to ACC with advancing maturity is further illustrated by the ethylene production responses of fruit of all nine maturity classes (Fig. 3-5B and inset). Relative to control (no ACC), the rate of ethylene production by fruit infiltrated with 1 mM ACC increased from 28- to 108-fold as fruit developed from class one to three. ACC-treated fruit produced as much as 50 nL C_2H_4 g f wt^{-1} h^{-1}, compared with a maximum of 1 nL g f wt^{-1} h^{-1} for control fruit, over the first three stages of maturity (Fig. 3-5B inset). Responsiveness to exogenous ACC then declined rapidly as fruit advanced in maturity beyond class three, so that the increase in ethylene production averaged only 7-fold in fruit of maturity classes six to nine. Vacuum infiltrating fruit of maturities one to nine with 1 mM SAM had no effect on the rate of ethylene production (Fig. 3-5C).

ACC treatment also hastened ripening of fruit (cv. Pembina) prior to harvest. Preharvest treatment of infructescences containing fruit of maturity class three with 1 mM ACC stimulated development to about maturity class eight within 5 days, while control fruit remained at maturity class three (Fig. 3-6, Table 3-1). Visible differences in maturity between control and ACC-treated fruit were first apparent by the third day of treatment and ripening from class four to eight only required an additional 48 h. (Fig. 3-6). Anthocyanin concentration of ACC-treated fruit was 5-fold greater than that of control (MES buffer) fruit after 5 days and was nearly equivalent to that of untreated fruit, which required 15 days to fully ripen from maturity class three (Table 3-1). On average, ACC-treated fruit gained 49% more fresh weight than control fruit over the 5-day treatment interval, but remained 2.6-fold smaller than the untreated fruit. Fruit color and size differences among these treatments were clearly evident (Fig. 3-7). Interestingly, fruit attached about 10 mm or more below the treatment point (cotton threads in Fig. 3-7B) did not ripen. Fruit attached either slightly below (5 mm or less) or even with the feeding point ripened to a lesser extent than fruit situated above the feeding point. This is indicative of a dominant, unidirectional transport of the exogenous ACC within the vascular system.

ACC synthase and ACC oxidase inhibitors were used to further assess the role of ethylene in saskatoon fruit ripening. 'Pembina' fruit, harvested at maturity class four and infiltrated with MES buffer (control), ripened to class seven within 3.5 days (Fig. 3-8). The rate of ethylene production by these fruit increased 42% from 0.5 days to 1.5 days after treatment, then decreased to 67% of the initial rate. Ethylene production rate of fruit infiltrated with 5 mM AOA was 3.2-fold less ($P<0.01$) than that of control fruit (averaged across days), while both treatments showed the same trend in ethylene production rate with time. Ripening of AOA-treated fruit was also delayed significantly relative to control fruit. AOA-treated fruit had only matured to class five after 3.5 days, compared with development to class seven for control fruit (compare fruit color in Fig. 3-8). Similar to results from the preharvest studies (Figs. 6, 7 and Table 3-1), 1 mM ACC effectively stimulated ethylene production by harvested fruit (compare control and ACC in Fig. 3-8); however, the change in production rate over time was significantly ($P<0.01$) different than that for control fruit. At 0.5 days after infiltration, the rate of ethylene production from ACC-treated fruit was 16- and 116-fold greater than that at 1.5 and 3.5 days, respectively (note log scale in Fig. 3-8). The inhibiting effect of AOA on ethylene production was eliminated when fruit were infiltrated with a combination of AOA and ACC. Ethylene production rate of these fruit was similar to that of ACC infiltrated fruit and both treatments stimulated ripening from stage four to seven within the 3.5 day interval. AVG, another inhibitor of ACC synthase, and Co^{++}, an inhibitor of ACC oxidase, were equally effective at inhibiting ethylene production and ripening of harvested, maturity class four 'Pembina' fruit (Table 3-2). Twenty four hours after infiltration, control fruit (MES buffer) produced ethylene at a rate that was 1.8-fold higher than that from inhibitor-treated fruit. AVG- and Co^{++}-treated fruit remained mostly green (maturity four), while control fruit ripened to maturity class six (red stage).

Discussion

Trends in ethylene production during ripening of saskatoon fruit (Figs. 3-2 and 3-3) were comparable to those of most climacteric fruits. As fruit advanced in maturity, endogenous ethylene levels rose (Fig. 3-3), concomitant with an increase in ethylene biosynthesis (Fig. 3-2). Biale and Young (1981) compared internal ethylene levels among climacteric fruits. At the peak of the climacteric, internal ethylene ranged from a low of 2.8 $\mu L\ L^{-1}$ for papaya to a high of 500 $\mu L\ L^{-1}$ for avocado. Banana, tomato, mango and plum contained 40, 27, 3 and 0.23 $\mu L\ L^{-1}$

ethylene, respectively, at their climacteric peaks (Tucker, 1993). In contrast, steady state ethylene levels for nonclimacteric orange, lemon and pineapple fruit ranged from lows of 0.1 to 0.3 μL L^{-1} to highs of 0.2 to 0.4 μL L^{-1} (Biale and Young, 1981). Maximum ethylene content of saskatoon fruit of various cultivars was anywhere from 1.2 to 3.3 μL L^{-1} (Fig. 3-3), well within the range of that for climacteric fruit and higher than that of most nonclimacteric fruit. Ethylene content of apple, a pome fruit belonging to the same family as saskatoon, was also variable depending on cultivar. Preclimacteric 'Idared' and 'McIntosh' fruit contained 0.1 μL L^{-1} ethylene which increased to 1 μL L^{-1} and 100 μL L^{-1}, respectively, during ripening (Chu, 1984; 1988). Changes in internal ethylene levels of saskatoon pomes were comparable with those of 'Idared' and 'Jonathan' (Chu, 1984; Kuai, 1992) apples.

For most cultivars of apple, ethylene production rates and internal ethylene levels increase progressively from physiological maturity onward and continue to increase after fruit have attained full ripeness (Chu, 1988; Watkins et al., 1989). In contrast, ethylene production rate of saskatoon cv. Northline fruit fell from a high at maturity nine (Fig. 3-2), while ethylene content of three of four other cultivars leveled off or declined over the latter stages of ripening (Fig. 3-3).

The increase in ethylene production and content during ripening of climacteric fruits is usually accompanied by an increase in respiration. On a fresh weight basis, respiration rate increased 60 to 100% during ripening of apple, 300 to 500% in tomato and banana, and 400 to 500% in avocado (Rhodes, 1970). In cranberry, however, an increase in ethylene occurred without a respiratory climacteric (Forsyth and Hall, 1969) and in *Cucumis melo* cultivars, respiration rate fell during ripening while ethylene levels increased (Miccolis and Saltveit, 1991). Respiration rates of ripening saskatoon fruit increased anywhere from 50% (cv. Northline) to 250% (cv. Smoky) on a whole-fruit basis during ripening (Figs. 3-2 and 3-3). When estimated on a dry weight basis, respiration rate of cv. Northline fruit only increased about 15% during the ripening phase (maturity class four onwards) (Fig. 3-2) and similar increases were difficult to resolve with any consistency in the other cultivars. Climacterics were also not apparent when respiration rates (fresh weight basis) of harvested fruit of each of the nine maturity classes were plotted against maturity class (Fig. 3-3 insets). This lack of increase in respiration likely reflects the tremendous increase in fresh weight that occurs concomitant with ripening. Fruit fresh weight doubles between maturity classes five and nine, while diameter increases linearly (Rogiers and Knowles, 1997). In many climacteric fruits maximum size is attained by physiological maturity, just prior to the increases in ethylene and respiration associated with ripening. Other changes involved in ripening then occur, either during (avocado, banana, mango) or after (apple, tomato) the respiratory climacteric (Rhodes, 1970). For saskatoon fruit, rates of fresh weight gain exceed rates at which whole-fruit respiration increases during ripening, resulting in downward trends in respiration rates on a fresh weight basis.

Anatomical studies of developing saskatoon fruit (Olson and Steeves, 1982; McGarry et al., 1997) showed that there are two major growth phases. The first phase spans ontogeny from postpollination through maturity class three and is characterized by extensive mitotic activity leading to an increase in the number of cells and cell layers. The second phase occurs from maturity classes three to nine, involves enlargement of the cells produced during phase one, and results in increased cell diameter, and number and size of intercellular spaces. Hence, cell number is determined relatively early in fruit development, well before the onset of ripening. Fruit respiration rate increased on a whole-fruit basis during ripening, at a time when cell number was constant (Figs. 3-2 and 3-3), reflecting increased respiratory metabolism per cell. Changes in respiration of saskatoon fruit during ripening are thus consistent with the increased metabolic activity associated with ripening of climacteric fruits in general. As indicated above, when respiration was expressed on a weight basis, changes in the basal metabolism of cells during ripening were masked by the substantive gains in water and carbohydrate content of fruit from maturity five through nine (Rogiers and Knowles, 1997). Unlike many other climacteric fruits, saskatoon fruit do not attain maximum size prior to ripening. This developmental characteristic is significant in that it precludes harvesting in the preclimacteric stage, a practice which is advantageous to postharvest handling and storage of many climacteric fruits.

It is interesting that the respiration rates of fully ripe 'Smoky' and 'Pembina' fruit were about 2- to 2.5-fold higher than those of 'Parkhill' and 'Thiessen' fruit on a fresh weight basis (Fig. 3-3 insets). On average, internal ethylene content of the high respiration rate cultivars was also about 2-fold greater than that of the low respiration rate cultivars (Fig. 3-3). Respiration rates have been positively correlated with ethylene production and content of fruits and vegetative tissues (Kader, 1987). Fruits with high respiration (e.g. strawberry, banana, cherry) generally tend to have shorter shelf lives (Wills et al., 1989). Because of their lower metabolic activity at maturity, 'Thiessen' and 'Parkhill' fruit may maintain postharvest quality longer and be better suited for fresh packaging systems than either 'Smoky' or 'Pembina' fruit.

Ripening of saskatoon fruit appears to be induced by ethylene and is therefore dependent on the ability of fruit to produce ethylene. The rate limiting step in ethylene biosynthesis in many tissues is the conversion of SAM to ACC by ACC synthase (Kende and Boller, 1981). Down regulation of ACC synthase through expression of an antisense ACC synthase gene in transgenic tomato fruit resulted in ethylene remaining below threshold levels for ripening (Oeller et al., 1991). Furthermore, ripening was inhibited until these transgenic fruit were treated continuously with exogenous ethylene. The increase in ethylene during ripening of apple (Bufler, 1984), pear (Knee, 1987), tomato (Brecht, 1987) and apricot (Sawamura and Miyazaki, 1989) has been correlated with increases in both ACC synthase and ACC oxidase. Preclimacteric apple fruit catabolized only 1% of the endogenous ACC per hour; however, during the climacteric the endogenous ACC pool was consumed every 30 min. (Knee, 1984). Similarly, abilities of preclimacteric apple (Hoffman and Yang, 1980; Yang, 1981; Dilley et al., 1995), avocado and muskmelon fruits (Hoffman and Yang, 1980) to synthesize ethylene from exogenous ACC were relatively low. These observations indicate that, for some fruits, ethylene biosynthesis in the preclimacteric stage may also be limited by low ACC oxidase activity (Ye and Dilley, 1992).

ACC oxidase was detected in saskatoon fruit of all maturity classes (Fig. 3-4). Levels of the enzyme on a protein basis increased substantially during development of 'Pembina' and 'Smoky' fruit, particularly during ripening (maturity classes four through nine). In contrast, ACC oxidase could not be detected by Western analysis in preclimacteric 'Golden Delicious' apple fruit (Dilley et al., 1993), and treatment of preclimacteric 'Paulared' apple with ACC did not stimulate ethylene production (Ye and Dilley, 1992). Unlike apple, ACC oxidase does not appear limiting to ethylene production by preclimacteric saskatoon fruit. Fruit of maturity classes one, two and three produced substantial amounts of ethylene when infiltrated with ACC (Fig. 3-5B). Furthermore, responsiveness to exogenous ACC was much less during the ripening stages (maturity classes four through nine), likely due to increased production of ACC by the fruit.

Ethylene biosynthesis by preclimacteric saskatoon fruit appears limited by the availability of ACC. Treatment of infructescences containing fruit of maturity class three with ACC hastened chlorophyll breakdown and stimulated anthocyanin biosynthesis and weight gain (Table 3-1, Fig. 3-7). Fruit within the ACC-treated infructescences had matured to class eight by 5 days after treatment, compared with little change in the maturation of control fruit (Figs. 3-6 and 3-7). In fact, maturity class three fruit from untreated infructescences required about 15 days to fully mature (Table 3-1, Fig. 3-7). Preclimacteric saskatoon fruit thus have adequate levels of ACC oxidase, along with the capacity to produce ethylene and ripen very early in development, but they evidently lack ACC, the immediate precursor of ethylene.

Limited ACC production in climacteric fruit prior to the onset of ripening has been attributed to a lack of ACC synthase (Cameron et al., 1979; Hoffman and Yang, 1990).

Treatment of saskatoon fruit with SAM failed to stimulate ethylene production (Fig. 3-5C), even though ACC oxidase was present (Fig. 3-4), suggesting that ACC synthase activity was limiting. However, the extent to which the exogenous SAM was utilized to synthesize other metabolites (e.g. polyamines) at the expense of ACC and ethylene is unknown.

Further evidence for the involvement of ethylene in ripening of saskatoon fruit was provided by ACC synthase and ACC oxidase inhibitor studies. Maturity class four fruit ripened to maturity class seven within 3.5 days of harvest and this was correlated with an increase in the rate of fruit ethylene biosynthesis (Fig. 3-8). AOA and AVG (ACC synthase inhibitors) significantly inhibited ethylene production and ripening of these class four fruit (Fig. 3-8, Table 3-2). Moreover, the inhibiting effects of AOA on ethylene biosynthesis and ripening were eliminated when fruit were treated with AOA plus ACC (Fig. 3-8). AOA inhibited lycopene synthesis in tomato pericarp tissue (Edwards et al., 1983) and our studies indicate that it also inhibits anthocyanin production, likely indirectly through an inhibition of ethylene biosysnthesis. This is in agreement with studies which have shown that ethylene promotes anthocyanin formation in fruits such as apple (Murphey and Dilley, 1988) and blackberry (Lipe, 1980). Inhibition of ethylene production by Co^{++} (ACC oxidase inhibitor) also inhibited ripening of saskatoon fruit (Table 3-2). Co^{++} effectively inhibited ethylene production by apple tissue (Lau and Yang, 1976).

In summary, we have shown that 1) respiration and ethylene production by saskatoon fruit increase during ripening, 2) immature fruit possess the ability to produce ethylene and ripen very quickly if given the ethylene precursor, ACC, 3) ACC synthesis is likely the rate limiting step in ethylene production by saskatoon fruit, and 4) inhibition of ethylene production significantly delays ripening. The results thus indicate that saskatoon fruit are climacteric, requiring ethylene to invoke and integrate their ripening response.

Fig. 3-1. Open airflow system for monitoring respiration of fruit as they develop on the plant. For clarity, only one chamber is shown; however, multiple chambers were connected by manifolds during an experiment. Individual fruit were sealed in a glass tube and airflow (2 mL/min) to each chamber was established through inlet and outlet syringe needles piercing the septum. The outflow from each chamber was directed through an infrared gas analyzer (IRGA) by a three-way solenoid valve controlled by a computer (interface not shown). Air from each chamber flowed through the IRGA for 45 min., whereupon the CO_2 concentration was recorded and the computer switched to the next valve, directing the outflow from the next chamber through the IRGA.

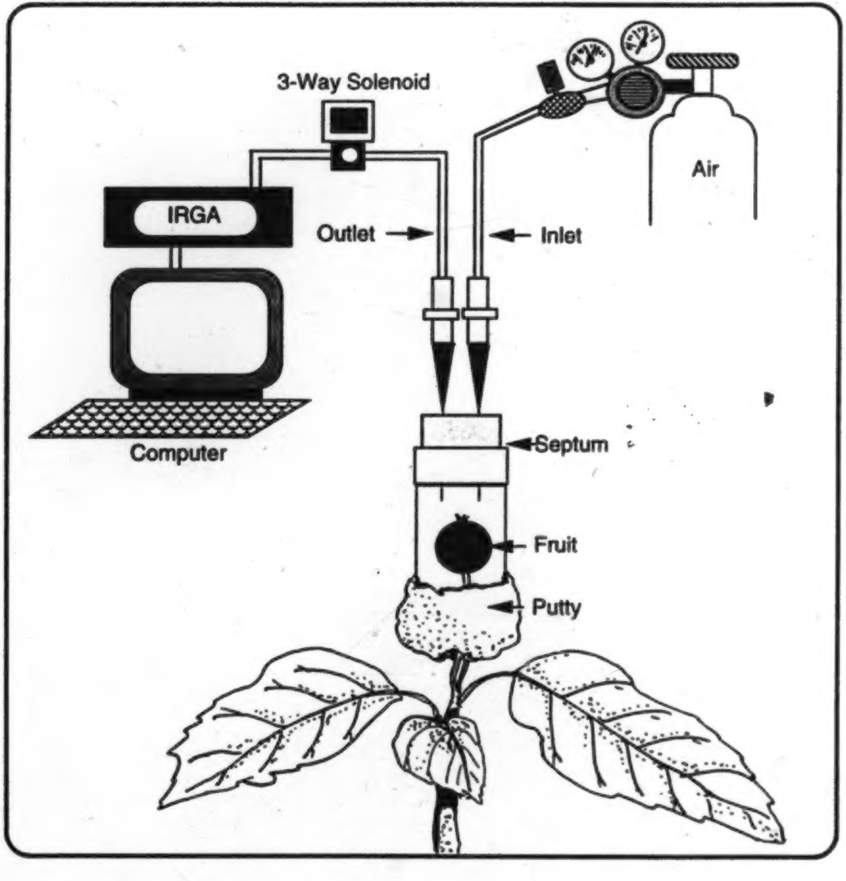

3-Way Solenoid
IRGA
Air
Outlet
Inlet
Computer
Septum
Fruit
Putty

Fig. 3-2. Respiration and ethylene production rates of saskatoon fruit (cv. Northline) during development on the plant. Numbers in brackets are saskatoon fruit maturity classes (see Rogiers and Knowles, 1997). Classes two, four, six and nine correspond to immature green, greenish-yellow, light red and dark purple, respectively.

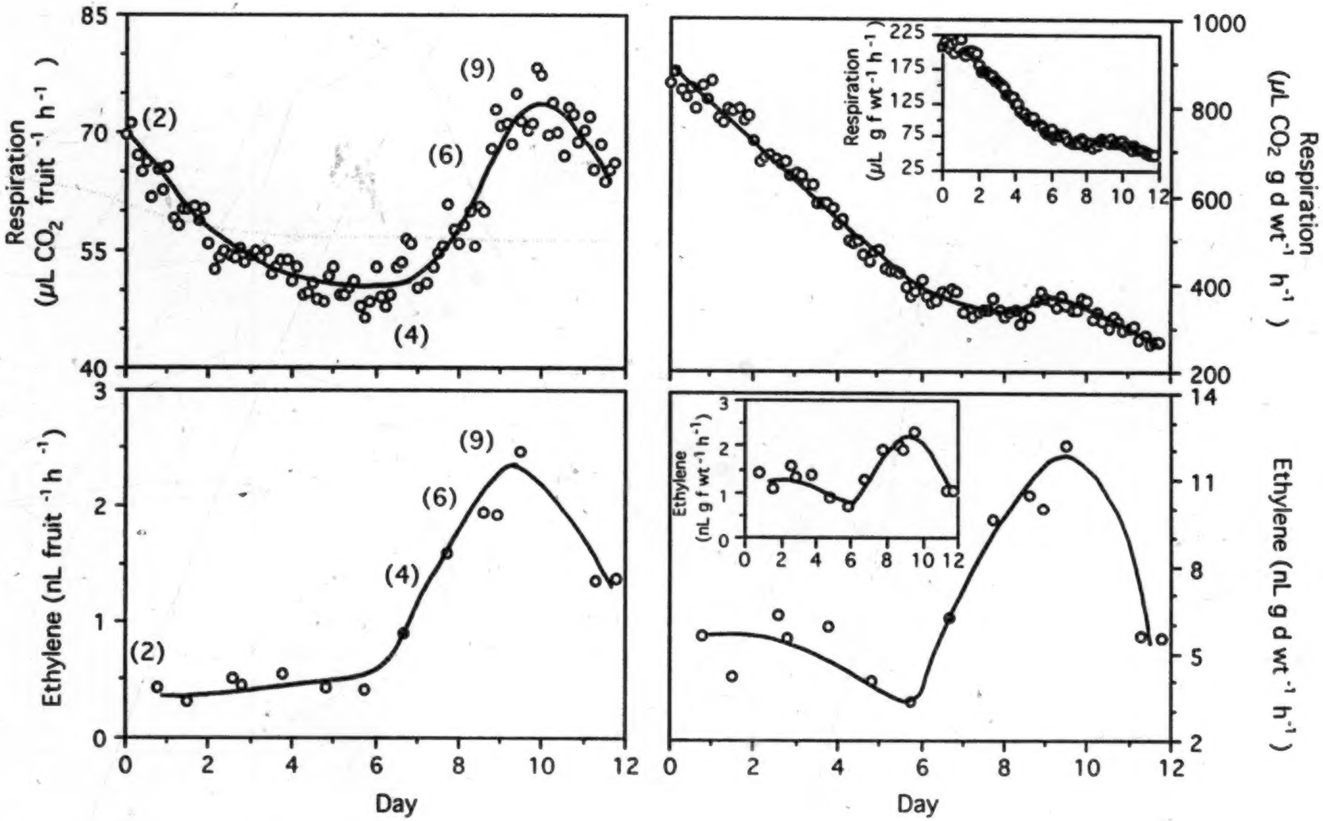

Respiration
(μL CO2 fruit -1 h-1)
85
70
55
40
(2)
(4)
(6)
(9)
Ethylene (nL fruit -1 h -1)
3
2
1
0
(2)
(4)
(6)
(9)
0
2
4
6
8
10
12
Day
Respiration
(μL CO2 g d wt-1 h-1)
1000
800
600
400
200
Respiration
(μL g f wt-1 h-1)
225
175
125
75
25
0 2 4 6 8 10 12
Ethylene (nL g d wt -1 h-1)
14
11
8
5
2
Ethylene
(nL g f wt-1 h-1)
3
2
1
0
0 2 4 6 8 10 12
0
2
4
6
8
10
12
Day

Fig. 3-3 Internal ethylene concentrations and respiration rates of harvested 'Thiessen', 'Parkhill', 'Pembina', and 'Smoky' fruit. Fruit were sorted into nine maturity classes using a maturity index system developed for saskatoon fruit (Rogiers and Knowles, 1997). Respiration rates were determined for individual fruit in an open air-flow system after 2 h acclimation at 23°C. Ethylene was extracted from 25 g samples of fruit under vacuum.

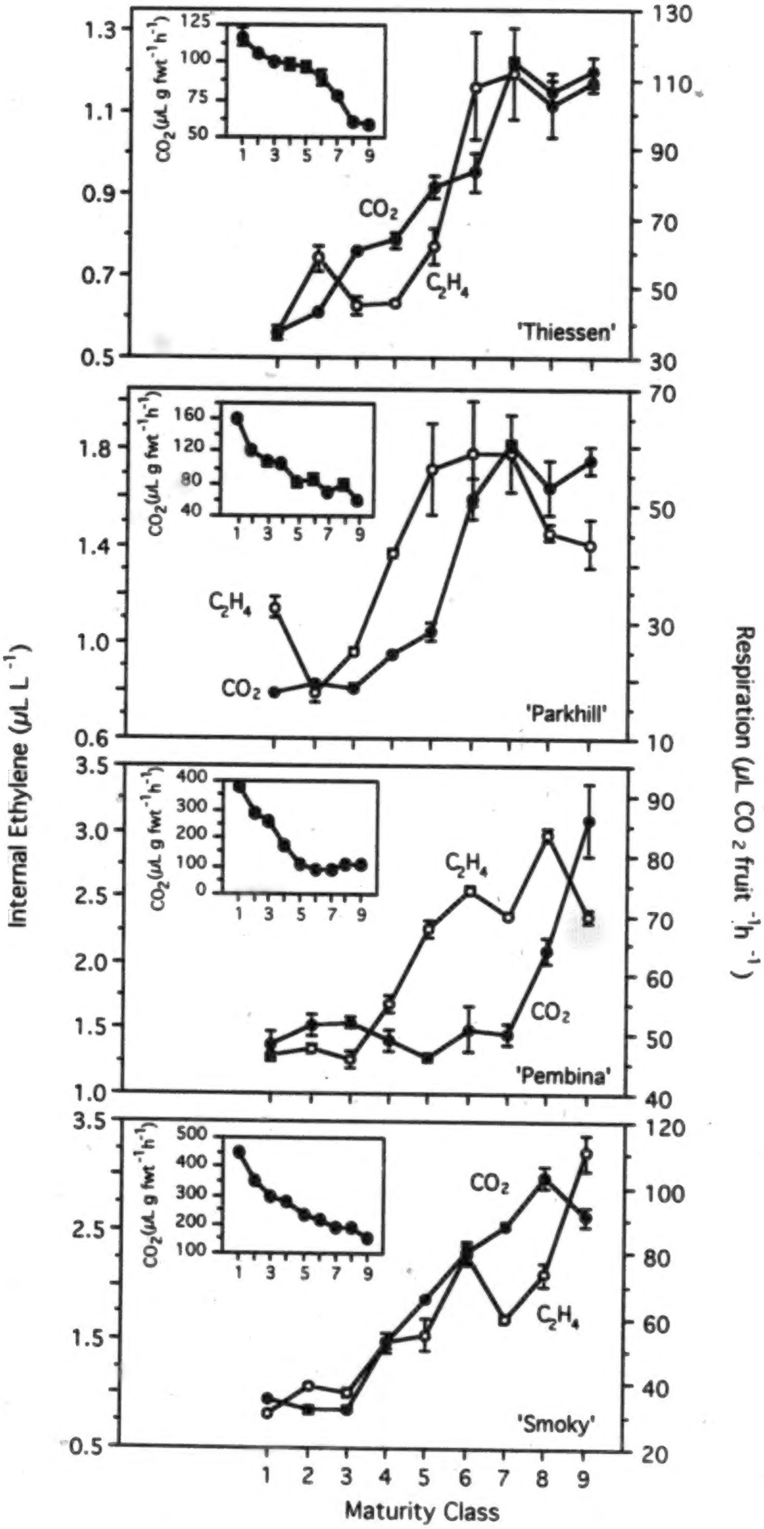

Internal Ethylene (μL L^{-1})
Respiration (μL CO$_2$ fruit^{-1}h^{-1})
Maturity Class
1 2 3 4 5 6 7 8 9
'Thiessen'
CO$_2$
C$_2$H$_4$
1.3 1.1 0.9 0.7 0.5
130 110 90 70 50 30
CO$_2$ (μL g fwt^{-1}h^{-1})
125 100 75 50
1 3 5 7 9
'Parkhill'
C$_2$H$_4$
CO$_2$
1.8 1.4 1.0 0.6
70 50 30 10
CO$_2$ (μL g fwt^{-1}h^{-1})
160 120 80 40
1 3 5 7 9
'Pembina'
C$_2$H$_4$
CO$_2$
3.5 3.0 2.5 2.0 1.5 1.0
90 80 70 60 50 40
CO$_2$ (μL g fwt^{-1}h^{-1})
400 300 200 100 0
1 3 5 7 9
'Smoky'
CO$_2$
C$_2$H$_4$
3.5 2.5 1.5 0.5
120 100 80 60 40 20
CO$_2$ (μL g fwt^{-1}h^{-1})
500 400 300 200 100
1 3 5 7 9

Fig. 3-4. SDS-PAGE of proteins (cv. Pembina), tissue print (cv. Pembina) and Western blots of ACC oxidase from nine maturity classes of saskatoon fruit. The tissue print (A) and Western blots (B, cv. Pembina; C, cv. Northline; D, cv. Smoky) were probed with anti-apple ACC oxidase. Protein (5 µg total load) from apple (cv. Spartan) was blotted (lane 1) alongside that from 'Northline' and 'Smoky' fruit. All saskatoon lanes were loaded with 80 µg of soluble protein.

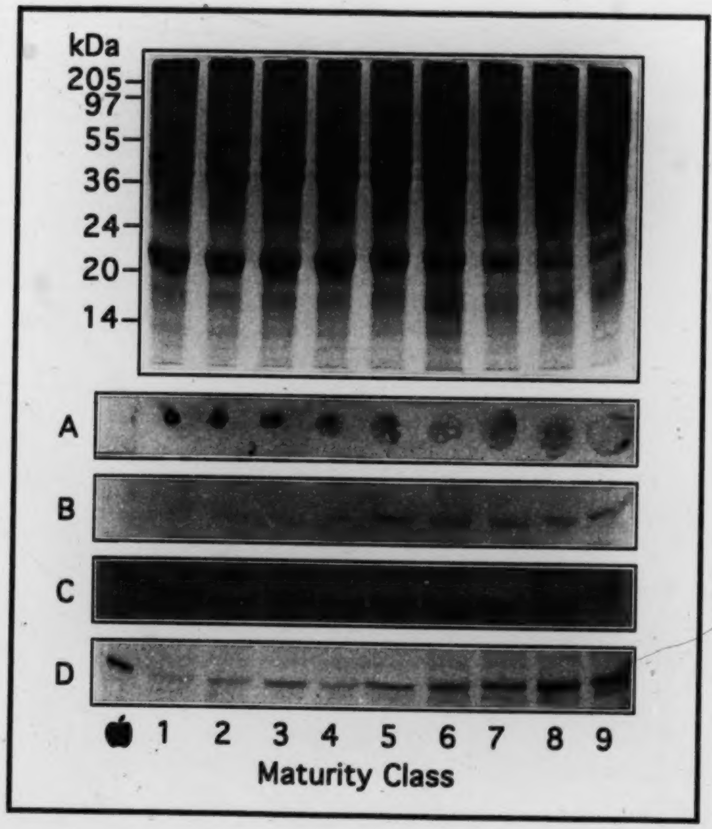
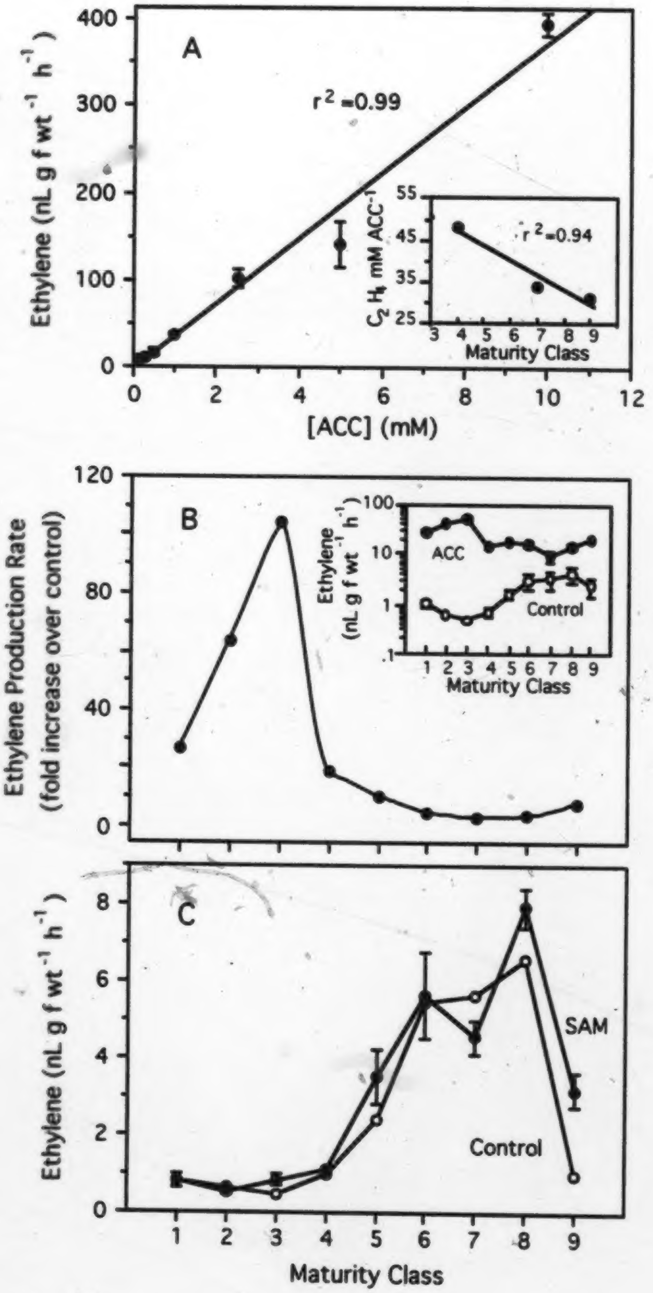

kDa
205
97
55
36
24
20
14
A
B
C
D
1
2
3
4
5
6
7
8
9
Maturity Class

Fig. 3-5. Effect of ACC and SAM on ethylene production by saskatoon (cv. Pembina) fruit of different maturity classes. Fruit of various maturity classes were vacuum infiltrated with ACC or SAM in MES buffer and ethylene production rates were determined in a closed system. Control fruit were infiltrated with MES buffer only. (A) Ethylene production rates of fruit (average of maturity classes four, seven, and nine) as effected by ACC concentration. Inset shows trend in ethylene production rate (nL g f. wt.$^{-1}$ h^{-1}) per mM ACC with advancing maturity class. (B) Increase in ethylene production rate obtained by infiltrating fruit with 1 mM ACC, as influenced by fruit maturity class. Inset shows the effects of ACC and maturity class on actual ethylene production rates (note log scale). (C) Effects of 1 mM SAM and maturity class on ethylene production rates of fruit.


A
r² =0.99
Ethylene (nL g f wt⁻¹ h⁻¹)
[ACC] (mM)
0
100
200
300
400
0
2
4
6
8
10
12
C2H4 mM ACC⁻¹
r² =0.94
25
35
45
55
3
4
5
6
7
8
9
Maturity Class
B
Ethylene Production Rate (fold increase over control)
0
40
80
120
Ethylene (nL g f wt⁻¹ h⁻¹)
ACC
Control
.1
1
10
100
1
2
3
4
5
6
7
8
9
Maturity Class
C
Ethylene (nL g f wt⁻¹ h⁻¹)
SAM
Control
0
2
4
6
8
1
2
3
4
5
6
7
8
9
Maturity Class

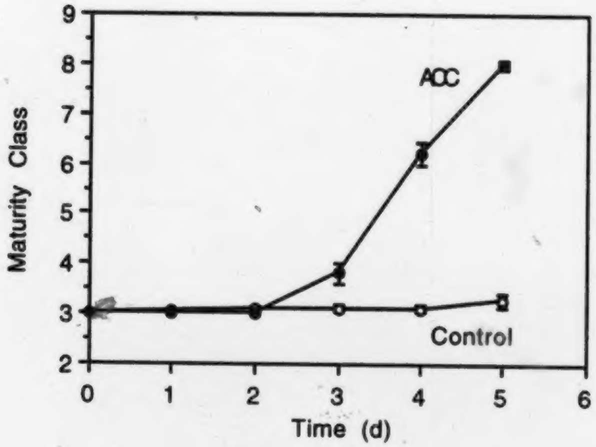


Fig. 3-6. Changes in maturity class of fruit (cv. Pembina) as affected by treatment with 1 mM ACC in MES buffer or MES buffer only (control). Treatment solutions were applied continuously over the 5 day interval to whole infructescences (developing on the plant) via a cotton thread that extended through the base of the peduncles (see Fig. 6). Infructescences contained eight to twelve fruit each, with an average maturity class of three (immature green) at zero-time (five replicate infructescences per treatment).

Fig. 3-7. Infructescences from saskatoon (cv. Pembina) plants showing the effect of ACC on ripening. Infructescences were treated (while growing on the plant) with either 1 mM ACC in MES buffer or MES buffer alone (control), via a cotton thread that extended through the base of the peduncle (arrow). Fruit within each infructescence averaged maturity class three (immature green) at zero-time and the infructescences were harvested and photographed after 5 days of continuous treatment. (A) Fruit below the treatment thread were removed. Untreated fruit required 15 days to develop to about the same maturity class as ACC-treated fruit. Fruit weight, anthocyanin concentrations and maturity classes are quantified in Table 1. (B) ACC-treated infructescences showing that fruit situated below the treatment thread did not ripen.

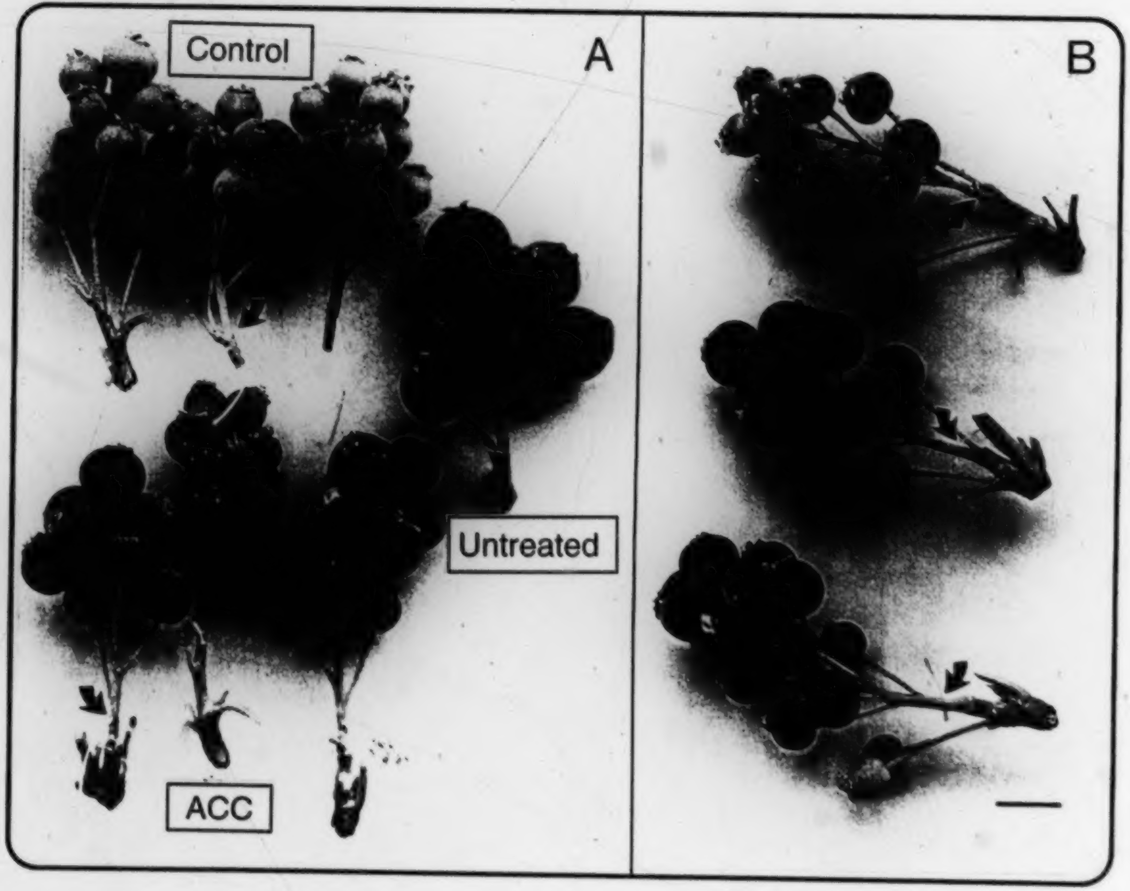

Control
A
B
Untreated
ACC

Table 3-1. Effects of preharvest treatment of infructescences with 1 mM ACC in MES buffer (50 mM, pH 7.0) on fruit growth and anthocyanin content. Infructescences had eight to twelve fruit which averaged maturity class three at treatment (see Figs. 3-6 and 3-7).

Treatment	Days to Harvest[a]	Fresh Weight (mg/fruit)	Anthocyanins (mg 100 g f wt^{-1})[b]	Maturity Class[c] at Harvest
MES buffer	5	229 ± 20	14.1 ± 1.6	3.3 ± 0.3
MES buffer + ACC	5	342 ± 62	72.8* ± 10.8	7.9** ± 0.2
None[d]	15	909 ± 121	86.8 ± 0.9	8.5 ± 0.2

[a] Assessed from time of treatment. [b] mg cyanidin-3-galactoside equivalents per 100 g fresh weight. [c] Estimated based on saskatoon maturity class index (Rogiers and Knowles, 1997). [d] No treatment indicates that fruit were left to ripen naturally, starting from maturity class three. *,**F-values for comparison of ACC with control (MES buffer) were significant at the 0.05 and 0.01 levels, respectively.

Fig. 3-8. Effect of ACC and AOA on ethylene production rate of saskatoon (cv. Pembina) fruit. Preclimacteric maturity class four fruit were harvested and vacuum infiltrated with MES buffer (control), AOA (5 mM), ACC (1 mM) or a combination of AOA and ACC (5 mM and 1 mM, respectively), all in MES buffer. Ethylene production rates were assessed at 0.5, 1.5, and 3.5 days after treatment. Photographs show maturity class four fruit at the time of treatment (day 0) and the relative ripening responses of fruit 3.5 days after applying the various treatments. In the 4 (chemical) x 3 (day) factorial ANOVA, F-values for the main effects of chemical and day, and the interaction of chemical x day were significant at the 0.01 level. Orthogonal comparisons further indicated that control vs. ACC and AOA vs. AOA+ACC were also significant at the 0.01 level. In a 2 (chemical) x 3 (day) factorial ANOVA, the main effects of control vs. AOA and day were significant at the 0.01 level, but the interaction was not significant.

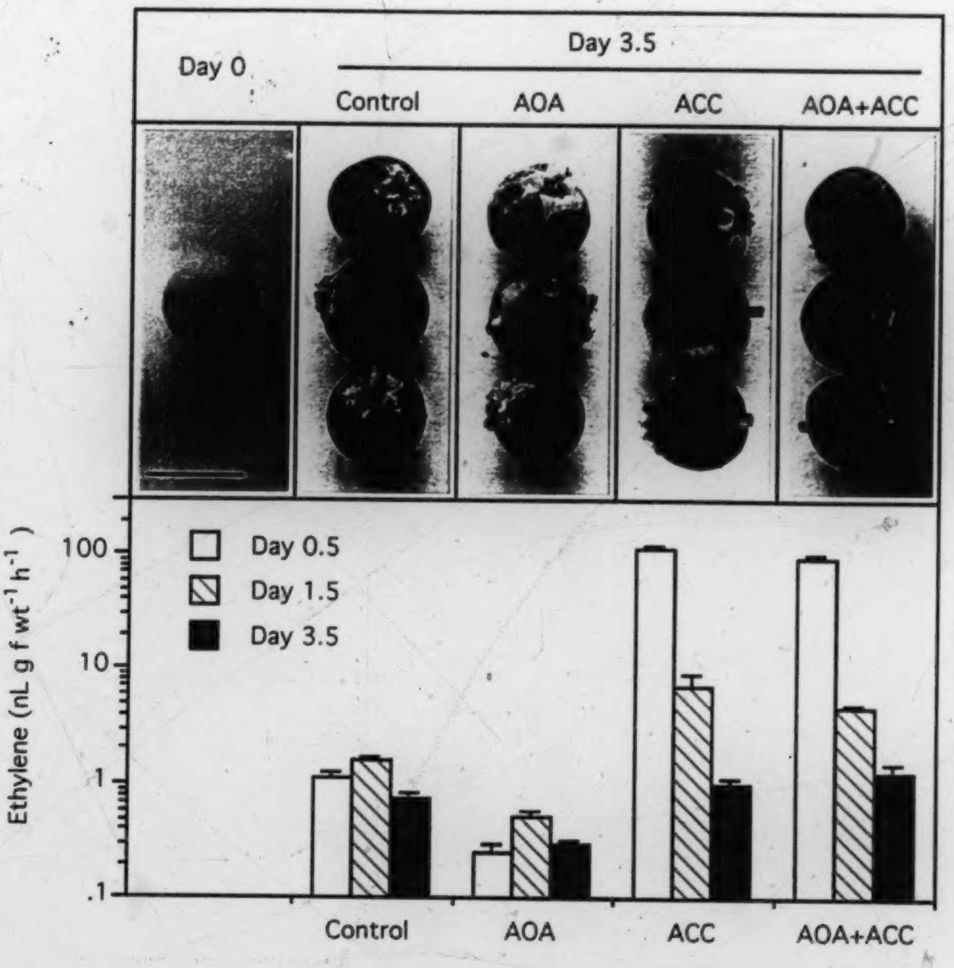

Day 0
Day 3.5
Control
AOA
ACC
AOA+ACC
Day 0.5
Day 1.5
Day 3.5
Ethylene (nL g f wt^{-1} h^{-1})
100
10
1
.1
Control
AOA
ACC
AOA+ACC

Table 3-2. Ethylene production and ripening responses of saskatoon (cv. Pembina) fruit 1 d after treatment with ACC synthase (AVG) or ACC oxidase ($CoCl_2$) inhibitors. Fruit, harvested at maturity class four, were infiltrated with the indicated inhibitor.

Treatment	Ethylene ($nL\ g\ f\ wt^{-1}\ h^{-1}$)	Maturity Class[a]
Control[b]	1.457 ± 0.103	6.3 ± 0.3
AVG (1 mM)	0.773 ± 0.146	4.6 ± 0.6
$CoCl_2$ (7 mM)	0.870 ± 0.071	4.6 ± 0.6
Control vs. Co^{++} + AVG[c]	0.01[d]	0.01
Co^{++} vs. AVG	NS	NS

[a]Estimated based on saskatoon maturity class index (Rogiers and Knowles, 1997). [b]All treatments in MES buffer (50 mM, pH 7.0). [c]Orthogonal comparison. [d]Significance level of comparison.

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Chapter IV

A Comparison of Preharvest and Postharvest Ethylene Production and Respiration Rates of Saskatoon (*Amelanchier alnifolia* Nutt.) Fruit During Development

Abstract

Postharvest changes in respiration and ethylene production rates of nine maturity classes of saskatoon fruit were compared with those of fruit maturing and ripening on the plant. During ripening on the plant, ethylene production increased on a whole-fruit and fresh weight basis, while respiration increased substantially on a whole-fruit basis but remained constant on a fresh weight basis. Fruit harvested at the greenish-yellow to pink stages (maturity classes four and five, respectively) increased ethylene production until 15-20 hours after harvest and this was coincident with ripening to maturity class seven and thus a color change to red. Ethylene production then declined over the next 15 hours. Respiration rates of harvested immature, mature, and ripe fruits declined over 5 days, except for a relatively brief 8 to 12% rise starting 15-20 hours after harvest. This increase in respiration was characteristic of fruit of all maturity classes, likely reflecting a wound response associated with harvesting rather than a ripening-induced change in metabolism. Continuous treatment of attached or detached fruit with propylene or ethylene in an open air-flow system did not induce the increase in respiration earlier than that from control fruit. The respiratory response of saskatoon fruit during postharvest ripening was thus unlike that of fruit ripening on the plant. An increase in respiration associated with ripening could only be demonstrated on a whole-fruit basis if fruit remained attached to the plant. Preharvest and postharvest changes in ethylene production rates during ripening also differed but were both consistent with climacteric ripening.

Introduction

During fruit development respiration rate per unit weight may steadily decline or, as in most climacteric fruits, may undergo a pronounced increase coincident with ripening (Biale and Young, 1981). The climacteric pattern of respiration was first described for apple (Kidd and West, 1925) and is characteristic of many fruits (e.g. mango, tomato, fig) (Burg and Burg, 1962; Lyons and Pratt, 1964; 1970; Marei and Crane, 1971). Intensity and duration of the respiratory climacteric varies widely among fruit species; however, it usually commences as fruit attain full size and before the characteristic changes in ripening (Wills et al., 1989). Relative to many other climacteric fruits, saskatoons are unusual in that fruit size doubles concurrent with the color, texture, and flavor changes associated with ripening (Rogiers and Knowles, 1997). Hence, though respiration increased on a whole-fruit basis during ripening on the plant, it remained relatively constant on a weight basis (Rogiers et al., 1997b).

Climacteric and nonclimacteric fruits can be further differentiated by their patterns of ethylene evolution and by their responses to applied ethylene. As is characteristic of climacteric fruits, saskatoons exhibit an increase in ethylene production rate and content during ripening on the plant. Furthermore, treatment of immature saskatoon fruit with 1-aminocyclopropane-1-carboxylic acid (ACC) induces ethylene production and hastens ripening, while inhibitors of ethylene biosynthesis delay ripening (Rogiers et al., 1997b). Ethylene thus has a central role in regulating the ripening of this fruit.

Most studies characterizing climacteric patterns of respiration and ethylene production during ripening have utilized harvested fruit. The postharvest behavior of fruit, however, does not always reflect that of fruit growing on the plant. For example, harvested muskmelon (Shellie and Saltveit, 1993) and tomato (Saltveit, 1993) exhibited a respiration climacteric, while attached fruit did not. The preharvest behavior of these fruits was thus similar to that characterized for saskatoons; namely, no preharvest respiration climacteric on a weight basis during ripening (Rogiers et al., 1997b). It was of interest to determine whether saskatoon fruit undergo a respiratory climacteric while ripening postharvest, similar to muskmelon and tomato. Ethylene production and respiration of harvested fruit were compared with that of fruit ripening on the plant. The pre- and postharvest responses of fruit to propylene and ethylene treatment were also characterized.

Materials and Methods

Preharvest respiration and ethylene production during fruit development

Three-year-old potted saskatoon (*Amelanchier alnifolia* Nutt., seedling selection) shrubs were acclimated for 1 week to room temperature under natural light supplemented with high pressure sodium lamps as described in Rogiers et al. (1997b). At the end of the acclimation period, immature fruit of maturity classes two to three (see below for definition of maturity classes) were enclosed individually in chambers while still attached to the plant (see Rogiers et al., 1997b) and a constant airflow of 2 mL min^{-1} was established through each chamber. The outflow from each chamber was directed through an LI-6262 CO_2/H_2O infra-red gas analyzer (LI-COR, Inc., Lincoln, Nebraska) by a three-way solenoid valve controlled by a computer (see Rogiers et al., 1997b). Carbon dioxide concentrations were recorded every 3 h for each fruit. Gas samples (1 mL) taken from the outlets were also analyzed for ethylene on a Photovac 10A10 Portable Photoionization GC (Photovac Inc., Thornhill, Ontario), equipped with a photoionization detector. Respiration and ethylene production rates were compared on a per fruit and fresh weight basis. Fruit weights in the chambers were derived as in Rogiers et al. (1997b). Changes in respiration and ethylene production with development were characterized for three cultivars, three plants per cultivar and at least four fruits per plant. A representative curve is shown (Fig. 4-1).

Preharvest respiration of fruit in the orchard

A closed chamber system was used to measure CO_2 production by 'Smoky' fruit in the field on day 177, 185, 192, and 198 of the year. These dates corresponded to fruit at maturity classes one, three, five and nine, respectively (Rogiers and Knowles, 1997; see below for description of each maturity class). A 250 mL erlenmeyer flask wrapped in aluminum foil was placed over a fruit (4 replicates per sampling day) and sealed around the peduncle with a longitudinally sliced rubber septum and an inert putty (Fun Tac™, LePage, Brampton, Ontario). One mL gas samples were withdrawn for CO_2 analysis after 15, 30, 45, 60, and 120 min. Carbon dioxide production was linear over this time period. Temperature within an adjacent flask was averaged over the sampling interval. Syringes were transported to the laboratory on ice and CO_2 was quantified with a Hewlett-Packard 5890A GC, equipped with a 2.4 m stainless steel column (3.2 mm o.d.) packed with HayeSep T (Hewlett Packard) and a thermal conductivity detector. The carrier gas (He) flow rate was 30 mL min^{-1} and the column was isothermal at 100°C. Injection and detector port temperatures were 140°C. Respiration rates ($\mu L\ CO_2\ h^{-1}$) were expressed on a per fruit and fresh weight basis. Rates were temperature-corrected to 24°C by the following equation:

$$R_{t_2}=R_{t_1} x\ Q_{10}{}^{((t_2-t_1)/10)}$$

where R_{t_1} was the rate of respiration at t_1 (actual temperature at sampling in the orchard); t_2 =24°C; Q_{10}=2.2, taken as the quotient (R_{t_2}/R_{t_1}) of the rates of respiration of apple fruit from 10 to 20°C (Forward, 1960).

Internal ethylene, ethylene production, and respiration of fruit after harvest

Saskatoon fruit, cvs. Northline and Smoky were hand harvested from 5 to 8 year old plants in a commercial orchard at Spruce Grove, Alberta. The fruit were sorted into nine maturity classes based on size and color, using an index system developed specifically for saskatoon fruit (Rogiers and Knowles, 1997). Fruit of maturity classes one to four were relatively immature, firm and green and contained high concentrations of chlorophyll. Chlorophyll content declined from stage four to nine, with a concomitant increase in anthocyanins. Fruit of maturity class five were 50% pinkish-red, six were light red, seven were dark red, eight were dark red with purple hues, and nine were purple. Fruit diameter increased linearly (r^2=0.99, $P<0.01$) at an approximate rate of 0.6 mm per maturity class, starting from an average of 7 mm for maturity class one (see Rogiers and Knowles, 1997)

To characterize postharvest changes in ethylene production, individual fruit (cv. Northline) of maturity class five were sealed inside 7 mL glass cylinders (3 cm long by 1.5 cm wide) with rubber septa. A continuous flow (2 mL min^{-1}) of humidified air (23°C) was then established through the chambers by hypodermic needles piercing the septa. Gas samples (1 mL), taken from the outflow from each chamber at various intervals over the 45 h postharvest study period, were analyzed for ethylene as described above. At the same intervals after harvest, endogenous ethylene was vacuum extracted (see Rogiers et al., 1997b) from 25 g samples (2 replicates) of cv. Northline fruit and quantified as they ripened from maturity class five over the 45 h postharvest period. In a parallel study, fruit harvested at maturity class four were photographed at various intervals over a 68 h period to document postharvest color development during ripening at 23°C.

Changes in respiration rates during ripening were compared among fruit (cv. Northline) harvested at maturity classes three, five, seven, and nine. Individual fruit (3 replicates) were placed inside 7 mL glass chambers at 23°C as described above and a humidified airflow of 20 mL min^{-1} was established through each chamber. The outflow from each chamber was directed through an LI 6262 infra-red gas analyzer (see above) and CO_2 was analyzed at 30 min intervals over a 35 h ripening period.

Preharvest and postharvest responses of fruit to ethylene and propylene treatments

Saskatoon plants (cv. Pembina) were grown as described in Rogiers et al. (1997b). Maturity class three fruit were enclosed individually in glass chambers while still developing on the plant (see Rogiers et al., 1997b) and, depending on the treatment, a constant flow (2 mL min^{-1}) of air or air containing 52 µL L^{-1} propylene was established through the chambers. The outflow from each chamber was directed through an LI 6262 infra-red gas analyzer and CO_2 was quantified every 6 hours for 12 days as fruit developed through maturity class nine. Respiration rates were expressed as $\mu L\ CO_2\ fruit^{-1}\ h^{-1}$ and the study was conducted at 23°C.

The respiratory responses of harvested maturity class five fruit (cvs. Northline and Parkhill) to exogenous ethylene at 23°C were also compared. Individual fruit were sealed inside 7 mL glass chambers that were continuously flushed (15 mL min^{-1}) with humidified air, or air containing 1 $\mu L\ L^{-1}$ or 5 $\mu L\ L^{-1}$ ethylene. Carbon dioxide was measured in the ouflow from each chamber with an infra-red gas analyzer (see above) at 30 min intervals over a 35 h and 45 h postharvest ripening period for 'Parkhill' and 'Northline', respectively. Respiration was calculated as $\mu L\ CO_2\ g\ fwt^{-1}\ h^{-1}$, expressed as a percentage of the respiration rate at time zero, and plotted against time.

To characterize the respiratory responses of fruit to different concentrations of exogenous ethylene over a longer postharvest period at 23°C, 10 g samples of maturity class five 'Smoky' and 'Northline' fruit (3 replicates) were placed inside 40 mL glass cylinders and flushed continuously (15 mL min^{-1}) with 0, 0.1, 1, 10, or 100 $\mu L\ L^{-1}$ ethylene in humidified air for 5 days. The outflow (1 mL) from each chamber was analyzed daily for CO_2. Carbon dioxide concentrations were quantified with a Hewlett Packard GC, as described previously. Respiration rates were expressed as $\mu L\ CO_2\ g\ fwt^{-1}\ h^{-1}$.

Results

Preharvest trends in respiration and ethylene production during development and ripening of saskatoon fruit were consistent with those reported previously and are characteristic of climacteric fruit (Rogiers et al., 1997b). Under controlled-environment conditions, a total of 15 days were required for fruit to develop from maturity class two to nine (Fig. 4-1). Ethylene levels remained below 1 nL $fruit^{-1}\ h^{-1}$ until day 8 (maturity class 3), then increased linearly to 5.5 nL $fruit^{-1}\ h^{-1}$. This trend was also evident on a fresh weight basis. The increase in ethylene occurred concomitant with a rise in whole-fruit respiration and began about 4 days before the onset of anthocyanin accumulation. Trends in respiration during ripening depended on how the rates were expressed. Respiration remained stable at 25 $\mu L\ fruit^{-1}\ h^{-1}$ over the first 9 days, then increased 2-fold to 55 $\mu L\ fruit^{-1}\ h^{-1}$ as fruit ripened to maturity class nine over the next 6 days (Fig. 4-1). On a fresh weight basis, however, there was a steady decline in respiration (to 25% of the original rate) until day 12 (maturity class 4), whereupon it remained constant at 50 $\mu L\ g\ fwt^{-1}$

h^{-1} as fruit ripened to maturity class nine over the next 6 days. At day 13 the fruit were still green (class four), they were red (class six) on day 14, and purple (class nine) by day 15.

Trends in respiration rates of fruit developing on plants under field conditions (i.e. in an orchard) (Fig. 4-2) were similar to those characterized under controlled-environment conditions. Respiration declined from 45 μL $fruit^{-1}$ h^{-1} (maturity one) to 32 μL $fruit^{-1}$ h^{-1} (maturity three), then increased to 61 μL $fruit^{-1}$ h^{-1} at maturity nine. On a weight basis, however, respiration declined steadily during development to 95 μL g fwt^{-1} h^{-1}, which was 40% of the original maturity class one rate.

Similar to the response displayed by fruit ripening on the plant, an increase in ethylene production was apparent during ripening of harvested fruit, and this coincided with a change from greenish-yellow (maturity class four) to red (maturity class seven) (Fig. 4-3). In a similar study, a 2.5-fold increase in ethylene content of maturity class five fruit took place within 15 h of harvest (Fig. 4-4). Fruit ethylene concentrations then declined rapidly over the next 15 h to near original values. Ethylene evolution also rose 3-fold until 20 h after harvest and then declined, mirroring the changes in fruit ethylene content (Fig. 4-4). To verify that the ethylene increase was associated with ripening and was not simply a consequence of wounding, changes in ethylene production rates by fruit of all nine maturity classes were compared over the first 33 h after harvest. At 4 h after harvest, maturity class six fruit (light red) had the highest rate of ethylene production of all classes, at 7 nL g^{-1} h^{-1} (Fig. 4-5). By 8 h after harvest, ethylene production rate of maturity class five fruit had increased from 3.5 to 6 nL g^{-1} h^{-1} and ethylene evolution from class six fruit had declined. The rate of ethylene production from maturity class five fruit reached a maximum of 10 nL g^{-1} h^{-1} at 18 h from harvest, and by 29 and 33 h it had declined significantly. No fruit of any of the other maturity classes produced ethylene above 4 nL g^{-1} h^{-1} from 8 to 33 h after harvest; therefore, the postharvest ethylene climacteric for cv. Northline fruit (Fig. 4-4) was unique to maturity class five, the stage at which color development began (Fig. 4-3). Changes in the ethylene production rates of harvested fruit (Figs. 4-4 and 4-5) were unlike those of attached fruit however, in that the latter had increasing rates of ethylene production with advancing maturity stages to full ripeness (Fig. 4-1).

The postharvest respiratory response was next examined by monitoring the rate of CO_2 production by fruit of maturity classes three, five, seven, and nine for 35 h after harvest. Respiration rate was calculated on a fresh weight basis and, while the absolute rates differed for each maturity class, the trends with time were similar. Fruit of all maturity classes experienced a small but consistent climacteric in respiration (Fig. 4-6). For ease of comparison among the maturity classes, respiration rates were plotted as a percentage of the rate at the beginning of the climacteric (equals 100) for each class (Fig. 4-6). Respiration rates declined more rapidly over the first 10 h after harvest for fruit of maturity classes three and five compared with those of classes seven and nine. The climacteric in respiration began about 13 h after harvest for fruit of all maturity classes. The maximum rates at 18 h were 8 to 12% greater than those at the beginning of the rise and respiration then declined over the remaining 17 h. This postharvest rise in respiration was thus not unique to maturity class five fruit, as was the postharvest increase in ethylene evolution. Furthermore, the relatively small increase in postharvest respiration, along with the fact that fruit of all maturity classes showed an increase of the same magnitude and at the same time following harvest, likely indicates that the response was not closely associated with ripening.

Respiration and ethylene production rates were also compared among maturity classes over a longer postharvest period (5 days) at 23°C. Respiration rates fell for fruit of all maturity classes, the decline being most rapid for immature (classes three, four and five) fruit, where rates reached half the original values (Fig. 4-7). Respiration of fruit harvested at maturity classes six through nine however, fell less than 30% over the 5 days, with the ripest fruit (maturity classes eight and nine) undergoing the slowest decline. Ethylene production rates by fruit of maturity classes three, four and five increased over the 5 day postharvest period. In contrast, ethylene production by maturity class six and seven fruit declined during the first 2 days after harvest, then increased through day 5. Changes in fruit fresh weight over the 5 day interval were negligible. Hence, trends in respiration and ethylene production rates on a per fruit basis were identical with those characterized on a fresh weight basis in Fig. 4-7. The changes in ethylene production and respiration rates of fruit while ripening after harvest were thus different than those exhibited by fruit ripening on the plant (for ethylene compare Figs. 4-4, 4-5 and 4-7 with Fig 4-1; for respiration compare Figs. 4-1 and 4-2 with Figs. 4-6 and 4-7).

The effects of ethylene and propylene, an analogue of ethylene that mimics its action, on the respiration of attached and detached fruit during ripening were also examined. In many climacteric fruits, the respiration climacteric is induced earlier in response to applied ethylene (or propylene) and the magnitude of the respiratory response is independent of the ethylene concentration above a threshold (Biale and Young, 1981). Saskatoon fruit were treated with 52 $\mu L\ L^{-1}$ propylene while developing on the plant, starting at maturity class three and continuing through full ripeness. The increase in respiration associated with ripening of propylene-treated fruit did not occur earlier than that of control fruit and the respiration responses were basically similar for both treatments (Fig. 4-8). Likewise, continuous treatment of harvested maturity class five fruit with 1 or 5 µL L^{-1} ethylene had no effect on timing or intensity of the small climacteric in respiration from 10 to 25 h after harvest (Fig. 4-9), providing further evidence that this postharvest response was independent of ripening-related metabolism. Exogenous ethylene treatment did, however, stimulate the overall respiration rates of harvested fruit and, although confounded by cultivar, the effect appeared to be greater for 5 µL L^{-1} than for 1 µL L^{-1} ethylene.

The concentration effect of ethylene on respiration rates of harvested maturity class five fruit was further defined in longer-term studies (Fig. 4-10). Concentration-dependent differences in respiration were well established by day 1 of a 5 day study in which fruit were continuously flushed with exponentially increasing concentrations of ethylene in air. The postharvest respiration rate of cv. Northline fruit was not affected by 0.1 and 1.0 µL L^{-1} ethylene, but increased in response to 10 and 100 µL L^{-1} ethylene from zero-time to day 1, and remained about 40% higher than that of control fruit through the remaining 4 days. From day 2 to day 5, the respiration rates of cv. Smoky fruit at 1, 10 and 100 µL L^{-1} ethylene were about 34%, 69% and 93% greater, respectively, than that of control fruit.

Discussion

Ethylene plays an integral part in the ripening of saskatoon fruit which are climacteric. Our studies have shown that exogenous ACC stimulates ethylene biosynthesis and ripening while ethylene biosynthesis inhibitors (AOA, cobalt, and AVG) effectively inhibit ethylene production and ripening (Rogiers et al., 1997). In this study, ethylene production by fruit developing on the plant increased on both a whole-fruit and fresh weight basis concomitant with ripening (Fig. 4-1). Fruit harvested at physiological maturity (i.e. the stage of development where fruit have the competence to ripen even if detached; about maturity class five for saskatoon fruit) exhibited an ethylene climacteric that coincided with the color changes associated with ripening (Figs. 4-3, 4-4 and 4-5). This postharvest increase in ethylene was not likely a consequence of wounding, since immature fruit (maturity classes one to four) and postclimacteric fruit (maturity classes eight and nine) showed no such response (Fig. 4-5). The increase in ethylene production by harvested maturity class five saskatoon fruit was comparable to that of detached ripening bananas, in which ethylene production reached a maximum of 14 nL g fwt^{-1} h^{-1} about 15 h after harvest and then declined (Inaba et al., 1989).

While ethylene was involved in the ripening of attached and detached saskatoon fruit (Rogiers et al., 1997b), there were differences in the trends and amounts of ethylene produced. Ethylene production rates of fruit of maturity classes eight and nine (nearly ripe to fully ripe) remained relatively low and constant after harvest (Figs. 4-5 and 4-7), as compared with attached fruit which showed a continuing increase (Fig. 4-1). As well, the change in ethylene production associated with ripening of detached class five fruit spanned only 24 hours (Figs. 4-4 and 4-5), while that of attached fruit continued for 2 days (Fig. 4-1). The most striking difference between pre- and postharvest ethylene production by ripening fruit was the climacteric change unique to harvested fruit.

The respiratory behavior of saskatoon fruit after harvest was unlike that of fruit developing on the plant. Respiration of attached fruit increased on a whole-fruit basis during ripening, while on a fresh weight basis it remained relatively constant (Figs. 4-1 and 4-2). In contrast, a small but consistent climacteric in respiration was evident for all maturity classes of fruit after harvest (Fig. 4-6). This small peak in respiration was not associated with ethylene production or ripening, as it was common among a wide range of maturity classes that displayed varying ethylene production rates (Fig. 4-4 and 4-5), abilities to undergo postharvest ripening, and times needed to ripen.

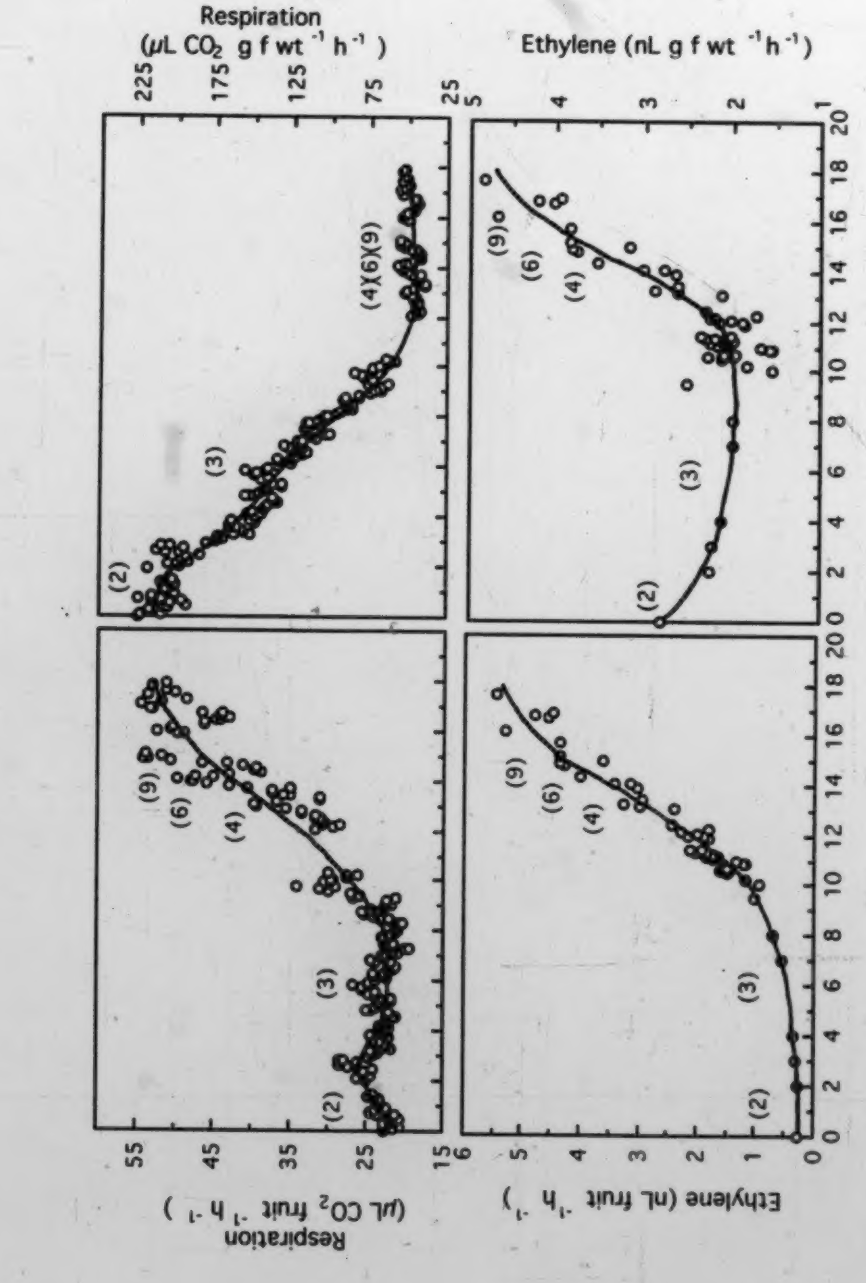
Many plant tissues respond to ethylene treatment with an increase in respiration rate (Sisler and Pian, 1973; Theologis and Laties, 1982; Duncan and Spencer, 1987; Esashi et al., 1987). In climacteric fruits, the onset of the respiration rise is usually shifted earlier in response to ethylene or propylene treatment (Biale and Young, 1981). Attached saskatoon fruit did not begin their ripening-associated increase in respiration earlier when treated with propylene, an ethylene analogue (Fig. 4-8), or ethylene (data not shown). This preharvest insensitivity of a climacteric fruit to ethylene is not unique, as tomato also appears to be resistant to ethylene-stimulated respiration until harvested (Saltveit, 1993). The small increase in respiration that consistently occurred around 10 h after harvest (regardless of fruit maturity at harvest) could not be induced earlier by ethylene treatment (Fig. 4-9). The magnitude of this postharvest increase in respiration rate was also unchanged by continuous treatment of fruit with ethylene. Exogenous ethylene did, however, stimulate the overall respiration rate of harvested fruit in a concentration-dependent manner (Figs. 4-9 and 4-10). This provides further evidence that the respiration rise of harvested fruit (Fig. 4-6) is not associated with ethylene or ripening.

The lack of a respiration climacteric on a fresh weight basis of attached fruit does not indicate that saskatoons are nonclimacteric. Respiration increased on a whole-fruit basis at a time when cell number had been determined. Hence, respiration per cell increased during ripening, even though the substantial increases in fresh and dry weights of fruit as they mature from class four to nine obscured the climacteric on a weight basis (Rogiers and Knowles, 1997; Rogiers et al., 1997b). The role of ethylene in the induction of ripening of saskatoon fruit has been confirmed (Rogiers et al., 1997b). As for the respiration increase, the general view is that it is not a criterion for ripening (Solomos, 1983; Tucker and Grierson, 1987; Tucker, 1993) but rather a byproduct of it. Hence, the critical difference between climacteric and nonclimacteric fruits is the requirement for and changes in the amount of endogenous ethylene during ripening (Reid and Pratt, 1970). As such, saskatoon fruit are best classified as climacteric.

The onset of saskatoon fruit ripening was hastened by harvesting (Fig. 4-3). This phenomenon was first reported for apple (Kidd and West, 1938) and has since been observed in avocado (Adato and Gazit, 1977), banana (Burg and Burg, 1965), peach (Looney et al. 1974), and citrus (Ben-Yehoshua and Eaks, 1970). The existence of certain 'tree factors' which act as anti-ripening compounds have been eluded to in other species (Burg, 1964; Sfakiotakis and Dilley, 1973), and it may be that these play a role in delaying the ripening of attached saskatoons. However, it is also possible that the stress induced by harvesting triggers the onset of ripening. Stressed, attached saskatoon fruit (e.g. by insect or pathogen invasion) often ripen earlier than their non-stressed counterparts. Similarly, we have observed increases in ethylene production and respiration rates upon fungal invasion of detached fruit (unpublished data). Ripening after harvest proceeded quickly, as is characteristic of climacteric fruit in general; however, ripening was often incomplete, as evidenced by a lack of full color development to purple. Competence of fruit to fully ripen after harvest depended on maturity class. Fruit of maturity classes five, six, seven, and eight were capable of complete color development and softening after harvest. Further studies to examine the extent of changes in other ripening parameters, such as flavor and volatile production, in fruit of these maturity classes would be of interest.

The results of these studies support the assertion by Saltveit (1993) that ethylene production and respiration patterns of harvested fruit are not necessarily the same as those of fruit ripening on the plant. Ethylene production increased on both a whole-fruit and fresh weight basis during ripening on the plant. Harvested maturity class five fruit showed an ethylene climacteric that lasted only until fruit reached maturity class seven. Respiration increased on a whole-fruit basis during ripening on the plant, but on a fresh weight basis it remained relatively constant. After an initial decline in respiration rate, harvested fruit of all maturity classes displayed a small respiration climacteric that was not closely associated with ripening.

Fig. 4-1. Respiration and ethylene production rates of saskatoon fruit (seedling selection) during development on the plant. Numbers in brackets are saskatoon fruit maturity classes (see Rogiers and Knowles, 1997). Classes three, four, six and nine correspond to immature green, greenish-yellow, light red and dark purple, respectively. An automated flow through system was used for monitoring respiration.


Respiration
(µL CO2 fruit -1 h -1)
55
45
35
25
15
Respiration
(µL CO2 g f wt -1 h -1)
225
175
125
75
25
Ethylene (nL fruit -1 h -1)
6
5
4
3
2
1
0
Ethylene (nL g f wt -1 h -1)
5
4
3
2
1
Day
0
2
4
6
8
10
12
14
16
18
20
(2)
(3)
(4)
(6)
(9)
(4)(6)(9)

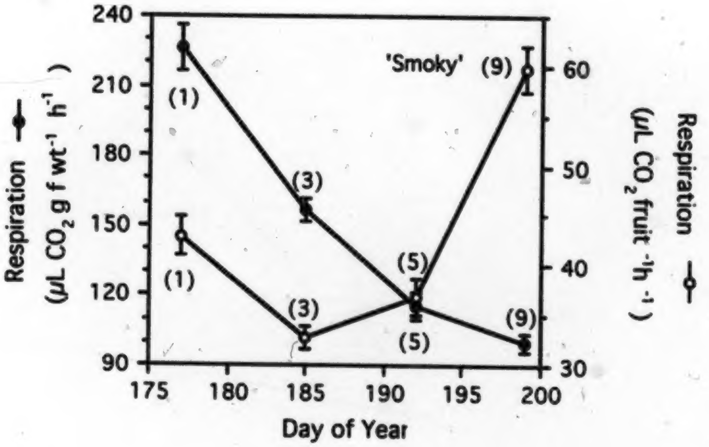
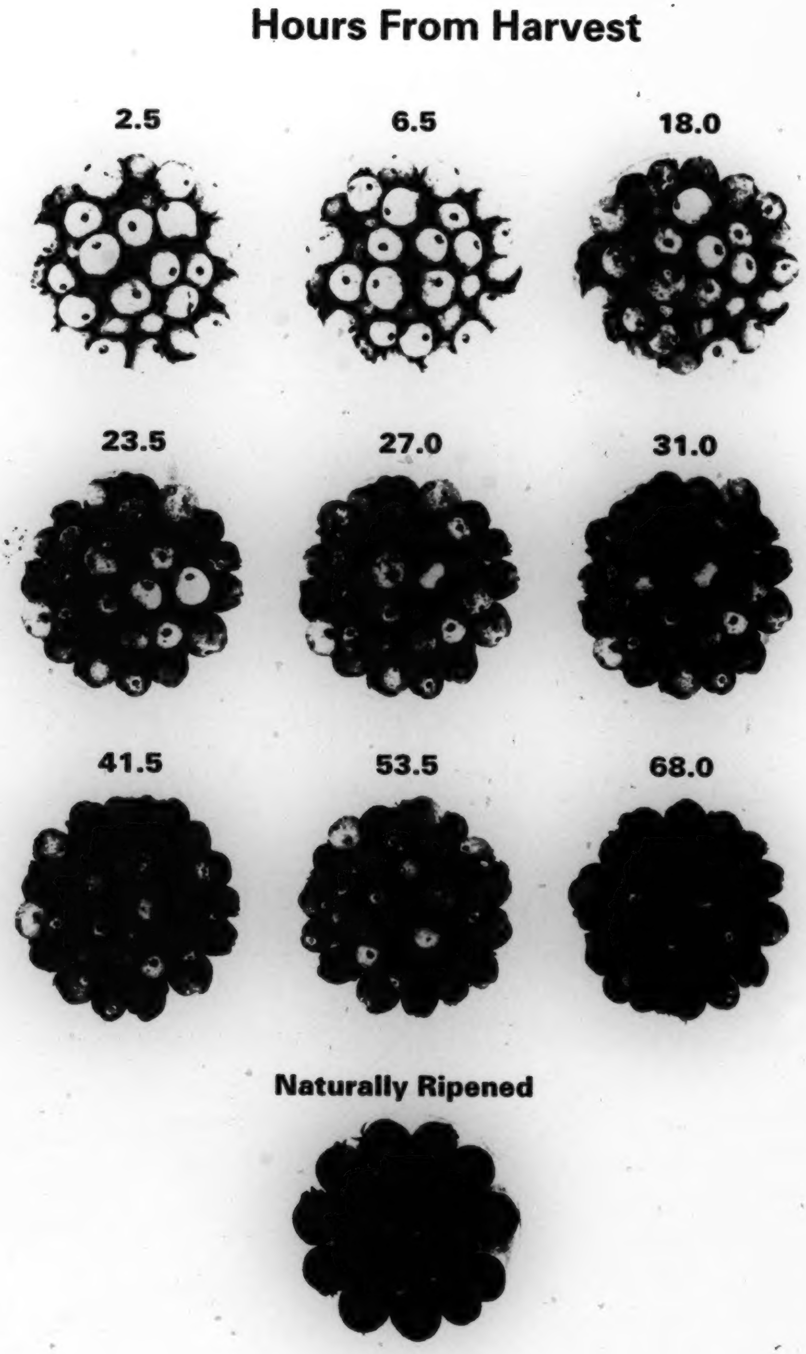


Fig. 4-2. Preharvest respiration rates of 'Smoky' fruit while developing in the orchard. Respiration rates were corrected to 24°C based on a Q_{10} of 2.2 (see Materials and Methods). Numbers in brackets indicate saskatoon fruit maturity class (see Rogiers and Knowles, 1997). Fruit of classes one and three were immature green, five were pink, and nine were dark purple.

Fig. 4-3. Color changes during ripening of harvested saskatoon cv. Northline fruit at 23°C. Fruit averaged maturity class four (greenish-yellow) at 2.5 h, class five at 23.5 h, class six at 41.5 h, and had developed to class seven (dark red) by 68 h after harvest. Fruit of maturity class nine (naturally ripened) were also harvested and are included for comparison (classes are defined in Rogiers and Knowles, 1997)


Hours From Harvest
2.5
6.5
18.0
23.5
27.0
31.0
41.5
53.5
68.0
Naturally Ripened

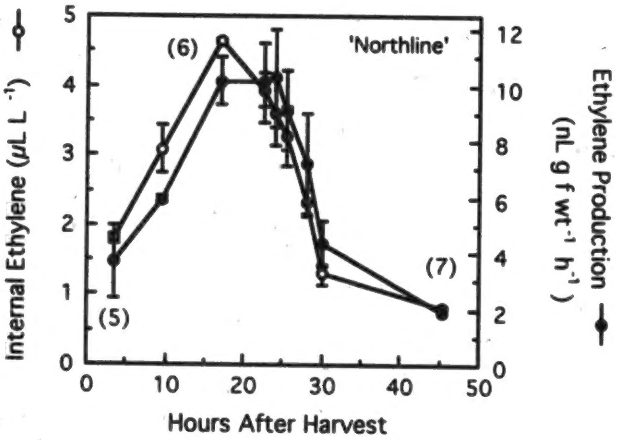
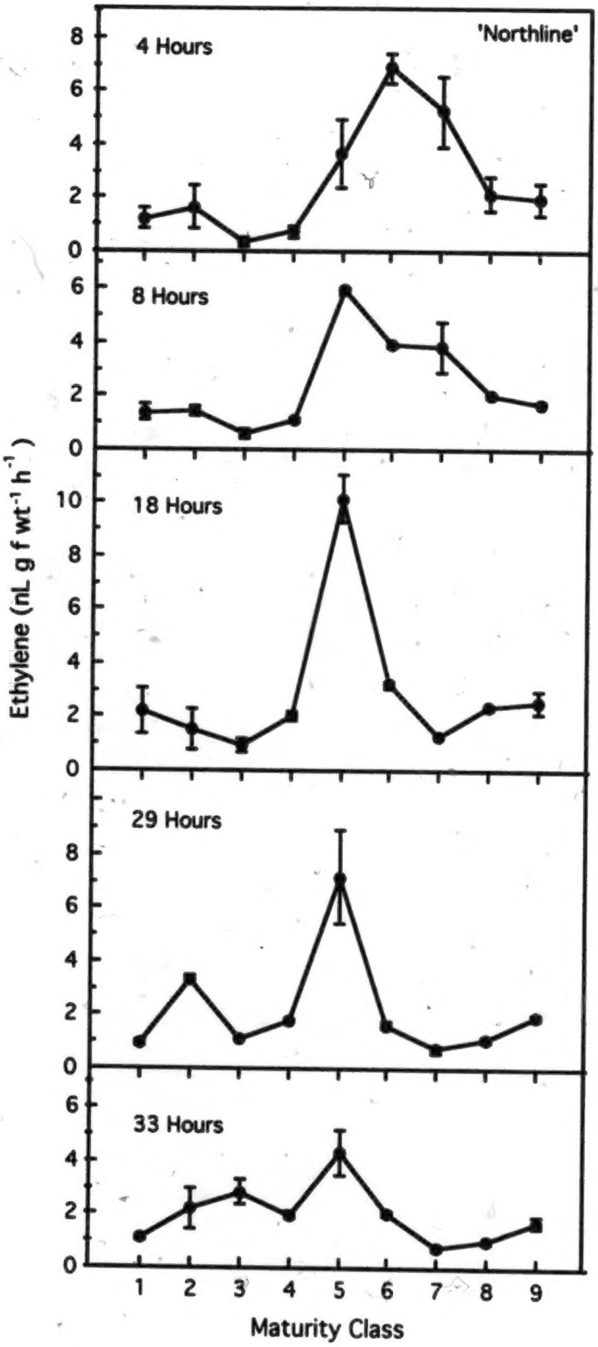


Fig. 4-4. Changes in internal ethylene concentration and production of harvested 'Northline' fruit. Fruit averaged maturity class five (50% pinkish-red) at harvest and were maintained at 23°C for 45 h in an open air-flow system. Numbers in brackets indicate fruit maturity classes (see Rogiers and Knowles, 1997). Fruit of classes six and seven were light red and dark red, respectively.

Fig. 4-5. Changes in ethylene production rates of 'Northline' fruit of maturities one to nine at 4, 8, 18, 29, and 33 hours after harvest. Fruit were sorted into nine maturity classes using an index system developed for saskatoon fruit (Rogiers and Knowles, 1997). Fruit of each maturity class were placed in an open air-flow system at 23°C and ethylene production was assessed over the 33 h postharvest period.


'Northline'
4 Hours
8 Hours
18 Hours
29 Hours
33 Hours
Ethylene (nL g f wt^{-1} h^{-1})
Maturity Class
0
2
4
6
8
10
1
2
3
4
5
6
7
8
9

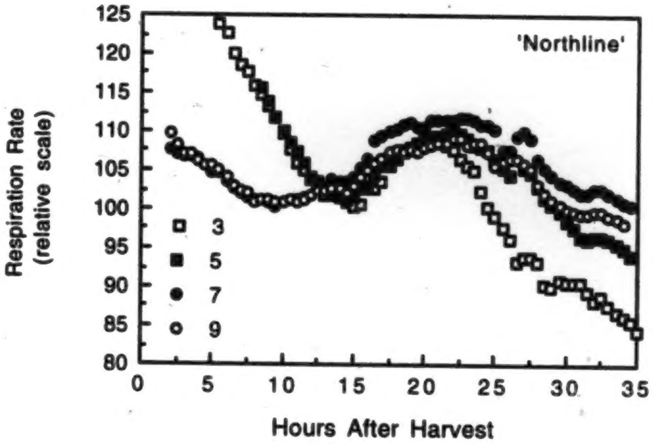
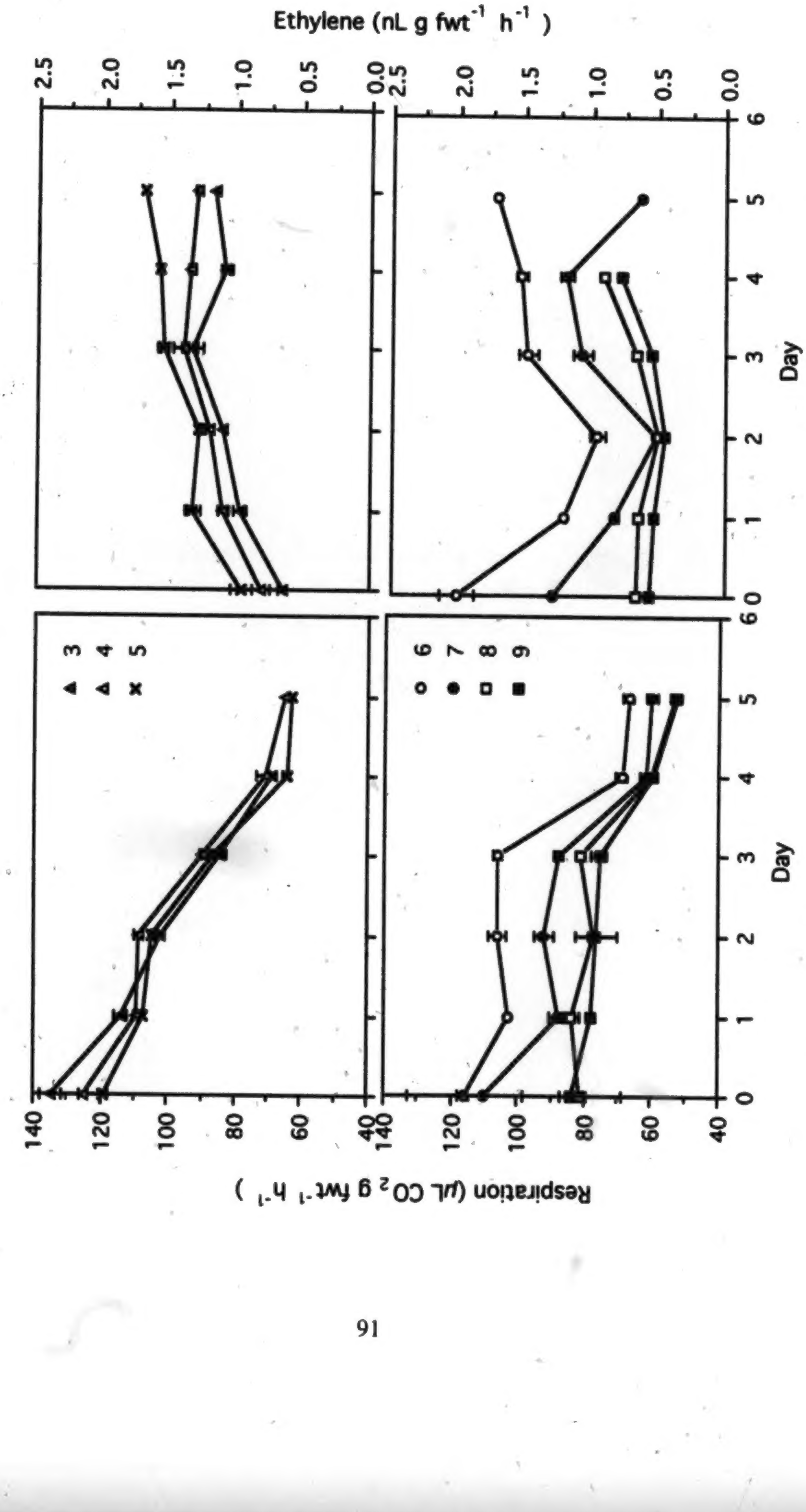


Fig. 4-6. Changes in respiration rates of 'Northline' fruit of maturity classes three, five, seven, and nine during a 35 hour postharvest period at 23°C. Respiration was monitored using an automated continuous flow system attached to a CO_2 analyzer. Rates (μL CO_2 g fwt^{-1} h^{-1}) are presented relative to those at the beginning of the postharvest rise (=100) for each maturity class. Maturity classes three, five, seven, and nine correspond to immature green, 50% pinkish-red, dark red and purple, respectively. Data presented are means of 3 replicates of individual fruit.

Fig. 4-7. Changes in respiration and ethylene production rates of 'Smoky' fruit of maturities three through nine (see Rogiers and Knowles, 1997) over a 5 day postharvest period. Fruit were placed in a flow through system at 23°C and gas samples were analyzed daily by GC. Ethylene data for fruit of maturity classes eight and nine at day 5 were lost due to GC malfunction.


Respiration (µL CO$_2$ g fwt^{-1} h^{-1})
Ethylene (nL g fwt^{-1} h^{-1})
140
120
100
80
60
40
2.5
2.0
1.5
1.0
0.5
0.0
0
1
2
3
4
5
6
Day
▲ 3
△ 4
× 5
○ 6
● 7
□ 8
■ 9

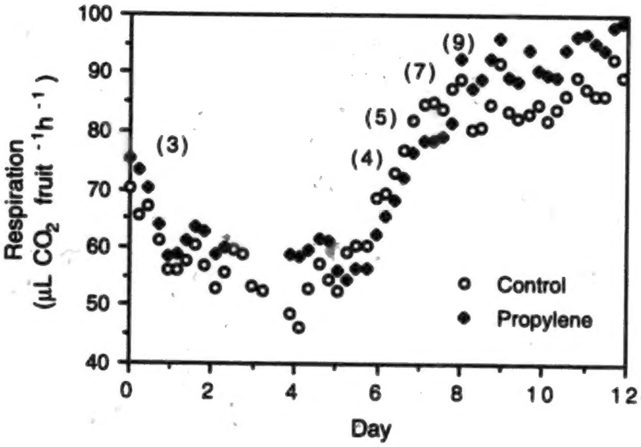


Fig. 4-8. Changes in respiration rates of cv. Pembina fruit while maturing on the plant as affected by a 52 µL L^{-1} continuous propylene treatment. Respiration was monitored at 23°C using an automated continuous flow system attached to a CO_2 analyzer. Fruit were of maturity class three at the outset of the study and were allowed to develop to an over-ripe stage. Numbers in brackets are saskatoon fruit maturity classes (see Rogiers and Knowles, 1997). Fruit of maturity class three were immature green, four were whitish-green, five were 50% pinkish-red, seven were light red, and nine were purple.

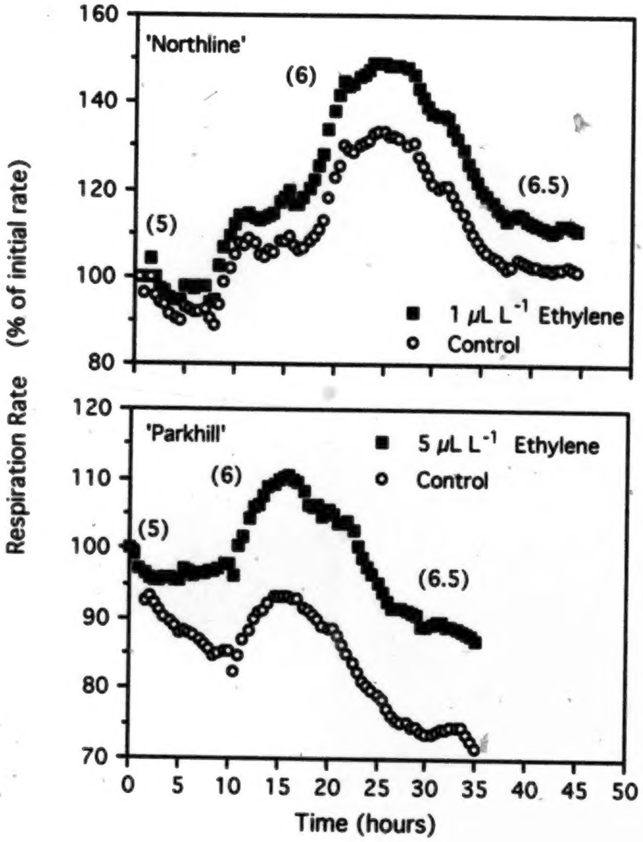


Fig. 4-9. Changes in respiration of maturity class five 'Northline' and 'Parkhill' fruit as affected by continuous treatment with ethylene during a 35 hour postharvest period at 23°C. Respiration was monitored using an automated continuous flow system attached to a CO_2 analyzer. Numbers in brackets are average saskatoon fruit maturity classes (see Rogiers and Knowles, 1997). Fruit of maturity classes five and six were 50% pinkish-red and light red, respectively.

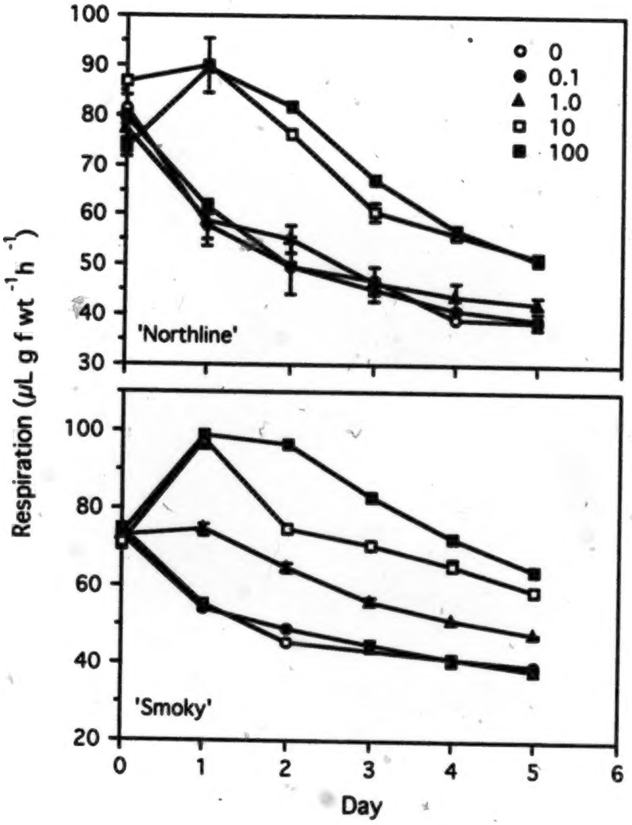


Fig. 4-10. Changes in respiration rates of harvested maturity class five 'Northline' and 'Smoky' fruit as affected by continuous treatment with 0 to 100 µL L^{-1} ethylene for 5 days. Fruit were placed in a flow through system at 23°C and gas samples were analyzed daily by GC. The ethylene concentration x time interaction was significant at the 0.01 level for each cultivar.

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Chapter V

Maturation and Ripening of Saskatoon (*Amelanchier alnifolia* Nutt.) Fruit are Accompanied by Increasing Oxidative Stress

Abstract

The extent of oxidative stress during ripening of saskatoon (*Amelanchier alnifolia* Nutt.) fruit was examined. Lipid peroxidation during fruit development from the mature green to the fully ripe (purple) stage was evidenced by the accumulation of ethane and 2-thiobarbituric acid reactive substances. Fruit polar lipid and free fatty acid concentrations also declined during ripening. Moreover, double bond index of polar lipid fatty acids fell during ripening, reflecting a progressive increase in the saturation of membrane lipids. This increase in saturation was partly due to a 65% decline in the concentration of linolenic acid. Activities of superoxide dismutase (SOD) and catalase (CAT) fell about 4-fold and 18-fold, respectively, during development, indicating higher potential for the accumulation of cytotoxic H_2O_2. Peroxidase activity remained relatively low and constant from the mature green to the dark red stage of development, then increased toward the end of ripening as fruits turned purple. Lipoxygenase (LOX) activity increased 2.5-fold from the mature green to the fully ripe stage. Tissue prints showed LOX to be present throughout fruit development and Western analysis revealed that the increase in activity during ripening was due to increased synthesis of the enzyme. Collectively, these results provide evidence that ripening of this climacteric fruit is accompanied by a substantial increase in free-radical-mediated peroxidation of membrane lipids, likely as a direct consequence of a progressive decline in the enzymatic systems responsible for catabolism of active oxygen species. The role of glutathione-mediated free-radical scavenging was also examined as a potential system for coping with this increased oxidative stress. Concentrations of reduced and oxidized glutathione (GSSG) increased 2-fold and GSSG increased as a percentage of total glutathione, reflecting the increase in oxidative status of fruits during ripening. Tissue prints of glutathione reductase (GRase) and transferase (GTase) showed these enzymes to be distributed throughout the pericarp at all stages of fruit development. GRase and GTase activities rose sharply during the latter stages of fruit ripening, correlating well with substantial increases in the levels of both enzymes. The glutathione-mediated free-radical scavenging system was thus up-regulated toward the end of ripening, perhaps in response to the

increasing oxidative stress resulting from the accumulation of lipid hydroperoxides from increased LOX activity, in conjunction with a decline in SOD/CAT activities.

Introduction

Increased free-radical mediated peroxidative damage and loss of membrane integrity are characteristics of senescing plant tissues (Dhindsa et al., 1981; Thompson, 1984). Like senescence, fruit ripening is accompanied by deterioration of cell membranes (Ferrie et al., 1994; Sacher, 1973) and the overall process may simply be a "...functionally modified, protracted form of senescence" (Huber, 1987). A loss of microsomal membrane integrity was observed during senescence of bell pepper (Lurie and Ben-Yehoshua, 1986) and during maturation and ripening of muskmelon (Lester and Stein, 1993) and apple fruits (Lurie and Ben-Arie, 1983). Similarly, ripening tomato fruit displayed increases in ion leakage and saturation index of membrane lipids, and the latter was attributed to a loss of linoleneate (Palma et al., 1995). Fatty acid unsaturation levels also fell in apple fruit during the postclimacteric stage of ripening (Lurie and Ben-Arie, 1983). While loss of membrane integrity during ripening is well documented, the early events leading to deterioration have yet to be characterized and the role of peroxidative mechanisms in the onset of this damage remain to be established.

If ripening is a protracted form of senescence, then the mechanisms of membrane deterioration during ripening are likely similar to those characterized in true senescing systems. Lipoxygenase (LOX) plays a central role in senescence-induced membrane deterioration by peroxidizing free polyunsaturated fatty acids (PUFA) (Paliyath and Droillard, 1992; Thompson, 1988). Specifically, LOX initiates oxidation of the cis,cis-1,4 pentadiene moieties present in PUFAs such as linoleic and linolenic acids (Todd et al., 1990; Ferrie et el., 1994; Droillard et al., 1993). The linoleyl-hydroperoxides thus formed decompose into oxy-free radicals, ethane and thiobarbituric acid reactive substances (TBARS), all of which are sensitive markers of lipid peroxidation. Both soluble and membrane associated LOXs have been identified in fruit, and were capable of the specific oxygenation of PUFAs esterified to phospholipids in the membrane bilayer (Droillard et al., 1993). Peroxidized PUFAs act as ionophores, admitting extracellular Ca^{+2}, which in turn enhances phospholipase activity, further cascading the deteriorative process (Leshem, 1987; Hildebrand, 1989).

Free radicals are generated as byproducts of normal metabolism and a decrease in the efficiency of their removal subjects cellular macromolecules and organelles to increasing oxidative stress. The glutathione cycle, along with superoxide dismutase (SOD), catalase (CAT) and various peroxidases, constitute the major enzymatic systems through which cells catabolize free radicals and H_2O_2, thus limiting the potential for oxidative damage. SOD catalyzes the dismutation of superoxide radical (O_2^-) to O_2 and H_2O_2. Peroxidase (POX) and CAT then catabolize H_2O_2 to H_2O and O_2 and hence limit the potential for further free radical production from H_2O_2. Peroxide and lipid hydroperoxides are also catabolized and thus detoxified by glutathione transferase (GTase). In the process, reduced glutathione (GSH) is oxidized (GSSG) and then converted back to GSH by glutathione reductase (GRase) using NADPH. Thus, glutathione levels and associated enzyme activities also play key roles in containing oxidative stress.

A gradual loss in ability to neutralize free radicals, due to reduced free radical scavenging by these enzyme systems, may be central to many of the metabolic changes associated with ripening. Lipid oxidation has been correlated with increasing peroxide levels of avocado, pear and tomato fruit tissues during ripening and senescence (Brennan and Frenkel, 1977; Brennan et al., 1979), and this may be a direct consequence of free radical buildup. The literature, however, is conflicting on this issue. For example, SOD activity decreased during senescence of leaves (Dhindsa et al., 1981) but increased during senescence of apple fruit (Du and Bramlage, 1994). In tomato fruit, SOD and peroxidase activities declined steadily from the immature green stage to the red-ripe stage (Rabinowich et al., 1982). This study provides an integrated overview of the extent of lipid peroxidation and associated oxidative stress during maturation of saskatoon (*Amelanchier alnifolia* Nutt.), a small pome fruit that exhibits climacteric-type ripening physiology (Rogiers and Knowles, 1996). Our results show that oxidative stress increases progressively during ripening, likely as a consequence of reduced activities of key enzymes responsible for quenching active oxygen species.

Materials and Methods

Fruit Sources and Maturity Classes

Depending on the study, saskatoon (*Amelanchier alnifolia* Nutt.) fruit were either produced in a controlled environment (cv. Pembina) or were harvested from a commercial orchard in Spruce Grove, Alberta (cv. Northline). Fruit from the orchard were frozen in liquid nitrogen at harvest and were stored at -30°C until further analysis. For production of fruit in growth chambers, dormant, 4-year old saskatoon shrubs were transplanted into pots in autumn and were vernalized at 4°C in the dark for 4 months. The plants were then transferred to a growth chamber set on a 12 h photoperiod with 14/8°C day/night temperatures under 450 μE m^{-2} sec^{-1} (at canopy top). Flowers were hand pollinated. Fruit were harvested, sorted into maturity categories based on a nine class index system (Rogiers and Knowles, 1997), and enzymes involved in oxidative stress (see below) were immediately extracted for analysis. The maturity classes spanned ontogeny of the fruit from immature (class one) to fully mature and ripe (class nine). Fruit of maturity classes one to four contained high concentrations of chlorophyll and thus were mostly green. Chlorophyll content declined substantially from stage four to nine, with a concomitant increase in anthocyanins, as fruit color changed from pink to purple. Fruit diameter increased linearly with advancing maturity class (Rogiers and Knowles, 1997).

Indices of Lipid Peroxidation

Ethane and thiobarbituric acid-reactive substances (TBARS) were quantified in developing fruit (cvs. Pembina and Northline, respectively) as indices of lipid peroxidation (Fletcher et al., 1973; Konze and Elstner, 1978; Dhindsa et al., 1981). At harvest, internal ethane was vacuum-extracted by submerging individual fruit in degassed water in a 10 mL syringe. The syringe was capped with a rubber septum, submerged and the plunger was withdrawn to the 10 mL mark and held for 2 min to extract gas from the fruit. A 300 µL gas sample was then analyzed for ethane. Ethane was separated and quantified on a Photovac 10A10 Portable Photoionization GC (Photovac Inc., Thornhill, Ontario) equipped with an 8' x 1/8" teflon column packed with Porasil B (100-150 mesh). The flow rate of the carrier gas (air) was 15 mL min^{-1}. Three replicates of individual fruit were used for maturity classes four through nine.

TBARS were extracted from the pericarp of frozen fruit of maturities four through nine and were quantified by modifying the methods of Heath and Packer (1968) and Dhindsa et al. (1981). Fruit (3 g fresh weight) was extracted in 6 mL of 0.1% (w/v) TCA by gently grinding (mortar and pestle) to avoid maceration of the seeds. The homogenate was filtered through Miracloth and centrifuged at 10,000 g for 20 min. All manipulations were at 4°C. The supernatant was diluted 100-fold, 100 µL was added to 0.9 mL of 20% (w/v) TCA containing 0.5% (w/v) TBA, and the mixture was incubated at 95°C for 30 min. Samples were then cooled on ice and centrifuged at 1640 g for 20 min. The samples had a relatively large A_{443} peak which was directly proportional (r^2=0.97) to a much smaller peak at A_{532}. Absorbance at 532 nm was consistent with that for a malondialdehyde (1,1,3,3-tetraethoxypropane) (Sigma) standard. Absorbance scans of blanks containing (1) TCA and TBA and (2) TCA and fruit extract (no TBA) revealed no absorbance at either of these wavelengths. Nonspecific absorbance of the samples at other wavelengths was also not evident. TBARS were thus expressed as A_{443} per g fresh weight.

Lipid Analysis

Three replicates of fruit of maturity classes four through nine were used for lipid extraction and analysis. Fruit were frozen in liquid N_2 in a mortar and cracked open with a pestle for removal of seeds. For each replicate, 5 g of tissue were ground to a powder and extracted with 45 mL chloroform:methanol:H_2O (8:4:1.5, v/v/v). Internal standards of phosphatidylcholine dipentadecanoyl (500 μg) and heptadecanoic acid (250 μg) were added to the extract. The solvent was filtered and transferred to a separatory funnel for phase separation. The lower phase was washed according to Folch et al. (1957) and evaporated to dryness.

The lipid residue and external standards of heptadecanoic acid and phosphatidyl choline were applied to thin layer chromatography plates coated with 0.5 mm silica gel G. The plates were developed in hexane:diethyl ether:acetic acid (8:20:1, v/v/v) to separate neutral lipids from polar lipids (phospho- and glycolipids). The plates were sprayed with 0.1% ethanolic 2',7'-dichlorofluorescein and the bands were visualized under UV light. The bands cochromatographing with heptadecanoic acid and phosphatidylcholine (origin of plate) were transferred to test tubes for transesterification and GC analysis of their acyl constituents.

Fatty acid methyl esters were prepared in 2% H_2SO_4 in methanol according to Christie (1989). These were separated on a Hewlett Packard 5830A gas chromatograph equipped with a flame ionization detector and a column of SP 2330 (15 m, 0.32 mm diameter) operated at 135°C. The detector and injector temperatures were 225°C and the carrier flow rate was 3.8 mL He min^{-1}. The GC was calibrated with 100 ng each of me-palmitate, me-stearate, me-oleate, me-linoleate, and me-linoleneate and quantitation was based on recovery of the internal standards of me-heptadecanoate (free fatty acids) and me-pentadecanoate (polar lipids). The double bond indices were calculated on mol percents as $DBI=[\%18:1+2(\%18:2)+3(\%18:3)]/(\%16:0+\%18:0)$ (Liljenberg and Kates, 1985).

Glutathione determination

Glutathione levels were analyzed in freshly harvested fruit (cv. Pembina) of maturities four through nine by the methods of Anderson (1985) and Smith (1985). Glutathione was extracted (mortar and pestle) from 3 g samples of fruit pericarp from each maturity class with 9 mL of 5% (w/v) sulfosalicylic acid at 4°C. The homogenate was filtered through Miracloth and centrifuged at 30,000 g for 20 min (4°C). The supernatant (600 μL) was neutralized with 900 μL of 0.5 M potassium phosphate buffer (pH 7.5) (Smith, 1985). Determination of oxidized glutathione (GSSG) was accomplished by first sequestering the reduced glutathione (GSH). This was done by incubating the diluted, neutralized extract with 20 µL of 2-vinylpyridine and 5 µL triethanolamine for 1 h at 23°C. Glutathione (GSSG or total) was assayed by the 5,5'-dithio-bis(2-nitrobenzoic acid)-GRase recycling method (Anderson, 1985). The reaction medium (2.0 mL) consisted of 1.6 mL sodium phosphate buffer (150 mM, pH 7.5) containing 24 mM DTNB, 4 mM NADPH, 1 Unit (final concentration) of GRase (Sigma) and 100 µL extract. The rate of increase in A_{412} due to TNB formation was determined with a Varian Cary 1E double-beam spectrophotometer at 30°C and glutathione levels were quantified by comparison with a standard curve of rates versus glutathione concentration. GSH levels were obtained by subtracting GSSG from total glutathione.

Enzyme extraction and assays

Enzyme extracts were prepared from the pericarp of freshly harvested fruit of maturity classes four to nine (cv. Pembina). Fruit (5 g fresh weight) was extracted in 5 mL of potassium phosphate buffer (0.1 M, pH 7.0) containing 0.05% (w/v) Triton X-100, 1 mM each of PMSF and EDTA, 5 mM DTT and 5% (w/v) PVPP by gentle grinding (mortar and pestle) to avoid maceration of the seeds. The homogenate was filtered through Miracloth and was centrifuged at 30,000 g for 15 min. The supernatant was desalted through a PD-10 Sephadex G-25M column (Sigma) and LOX, POX, CAT, GTase and GRase activities were determined on the eluant as outlined below. For extraction of SOD, 3 g of fruit were ground in 12 mL of potassium phosphate buffer (50 mM, pH 7.3) and the homogenate was filtered and centrifuged (as above). SOD activity was determined in the supernatant. All manipulations were at 4°C.

Cytosolic LOX activity was measured at 30°C by monitoring the formation of conjugated dienes from linoleic acid at A_{234} (Shimizu et al., 1990). The reaction medium (1 mL) contained 790 µL potassium phosphate buffer (0.1 M, pH 7.0), 100 µM linoleic acid (final concentration) and 200 µL of enzyme extract (1.5 to 3.0 µg protein μL^{-1}). LOX activity was expressed as ΔA_{234} min^{-1} mg^{-1} protein.

SOD activity was determined by quantifying the ability of enzyme extracts to inhibit light-induced conversion of NBT to formazan (Beauchamp and Fridovich, 1971). The reaction medium (3 mL) consisted of potassium phosphate buffer (50 mM, pH 7.8) containing 13 mM methionine, 75 mM NBT, 2 mM riboflavin, 0.1 mM EDTA and 50 to 150 µL of enzyme extract. The reaction proceeded under high-intensity sodium vapour lamps (400 µE $m^{-2}s^{-1}$) at 25°C for 15 min and was halted by transferring the samples to darkness. Sample A_{545} was compared to a standard curve of percent inhibition of color development versus volume of enzyme extract and one unit of SOD activity was equal to the volume effecting 50% inhibition. Activity was expressed as units mg^{-1} protein.

CAT and POX activities were assayed at 30°C as described by Chance and Maehly (1955) and Soressi et al. (1974), respectively. The reaction medium for CAT consisted of 100 mM sodium phosphate buffer (pH 6.5) containing 12 mM H_2O_2 and 25 μL of enzyme extract in a total volume of 1 mL. The rate of catabolism of H_2O_2 by CAT was expressed as ΔA_{240} min^{-1} mg^{-1} protein. The POX reaction medium consisted of sodium acetate buffer (50 mM, pH 6.0) containing 15 mM guaiacol, 0.03% (v/v) H_2O_2 and 25 μL of enzyme extract in a total volume of 1 mL. Enzyme activity was expressed as ΔA_{470} min^{-1} mg^{-1} protein.

GRase activity was determined at 30°C by adding 100 μL of extract to 100 mM potassium phosphate buffer (pH 7.0) containing 2 mM GSSG, 150 μM NADPH (prepared in 0.1% (w/v) $NaHCO_3$), and 1 mM EDTA in a total volume of 1 mL. Oxidation of NADPH by GRase was monitored at 340 nm and the rate (nmol min^{-1}) was calculated using the extinction coefficient of 6.2 mM^{-1} cm^{-1}. GTase activity was also determined in a total volume of 1 mL at 30°C. The reaction medium consisted of potassium phosphate buffer (100 mM, pH 6.5) containing 2 mM each of GSH and 1-chloro-2,4-dinitrobenzene, and 100 μL enzyme extract. The rate of formation of S-2,4-dinitrophenylglutathione (a GSH-1-chloro-2,4-dinitrobenzene conjugate) by GTase was quantified at 340 nm using the extinction coefficient of 9.6 mM^{-1} cm^{-1} (Habig and Jacoby, 1981; Warholm et al., 1985). GRase and GTase activities were expressed on a protein basis. In all studies, fruit soluble protein was determined by the BCA method (Stoscheck, 1990) using protease-free BSA as a standard.

SDS-PAGE and Western Analysis

Samples of extracts from the LOX, GRase and GTase studies were diluted 1:1 with SDS-buffer [62.5 mM Tris (pH 6.8) containing 4.9% (v/v) glycerol, 0.97% (w/v) SDS, 4.9% (v/v) β-mercaptoethanol and 0.002% (w/v) bromophenol blue] and incubated at 95°C for 10 min. Proteins were then separated by SDS-PAGE on a 10% running (4% stacking) gel (Laemmli, 1970). Proteins in the gels were stained with Coomassie blue R 250 or were electroblotted (100 V for 1 h) to nitrocellulose for Western analysis. Anti-potato LOX (Geerts et al., 1994), anti-spinach GRase (Tanaka et al., 1994) and anti-*Hyoscymus muticus* GTase (Bilang and Sturm, 1995) were diluted

1:1000 with blocking buffer and were used to probe LOX, GRase and GTase levels on the blots. Immunoblots were developed with alkaline phosphatase-conjugated secondary antibody (see Kumar and Knowles, 1996).

Tissue Printing

Freshly harvested cv. Pembina fruit of maturity classes one to nine were halved transversely and placed cut side down on nitrocellulose paper. The fruit halves were removed after 1 min and the prints were allowed to dry. Tissue prints were blocked for 1 h with phosphate buffer saline [10 mM NaH_2PO_4 buffer (pH 7.2) containing 150 mM NaCl, 0.3% (w/v) Tween 20] which also removed anthocyanins and other fruit phenolics from the blot. The prints were then incubated for 2 h with antibodies against LOX, GRase and GTase and developed as described above. All manipulations were at room temperature.

Results and Discussion

Amelanchier alnifolia Nutt. produces a small pome fruit, about 1.0 to 1.5 cm in diameter, that is borne on shrubs in racemelike inflorescences. Fruit growth of several cultivars has been modeled (McGarry et al., 1997) and a nine-stage maturity index system based on fruit size, color and chemical composition was recently developed (Rogiers and Knowles, 1997) to facilitate further studies on the physiology and biochemistry of ripening. In this index system, early developmental stages (maturities one to four) are characterized by relatively slow fruit growth, the fruit remaining green and firm. During the ripening phase (maturities five to nine), fruit weight and diameter more than double, anthocyanins accumulate, chlorophyll breaks down, volatiles are produced and softening occurs (Rogiers and Knowles, 1997). Saskatoon fruit also exhibit climacteric-type ripening physiology (Rogiers and Knowles, 1996). Rates of ethylene production and respiration increase substantially as fruits mature from class five to nine, with timing of the maxima dependent upon cultivar. Moreover, treatment of preclimacteric fruit with ACC induces ethylene production and stimulates ripening. Ripening is accompanied by substantial increases in concentrations of reducing sugars and sorbitol, while the major organic acids malate and succinate decline (Rogiers and Knowles, 1997). In general, ripening of saskatoon fruit entails a multitude of physico-chemical changes, many of which are catabolic in nature. The extent to which oxidative stress accompanies and perhaps contributes to these changes was the focus of these studies.

Changes in oxidative stress during fruit development can be assessed by the extent of lipid peroxidation. Membrane lipids and free fatty acids are highly susceptible to oxidation and increasing oxidative stress is thus indicated by the accumulation of lipid peroxidation products. Ethane and TBARS are produced from the spontaneous decomposition of lipid hydroperoxides and thus are regarded as sensitive markers of peroxidative damage (Fletcher et al., 1973; Riely et al., 1974; Konze and Elstner, 1978). Concentrations of ethane and TBARS increased in saskatoon fruit during development (Fig. 5-1). Ethane increased from undetectable levels in maturity class six fruit (light red stage) to 300 ppb as fruit developed to maturity class nine (dark purple stage). The increase in TBARS was 3-fold on a fresh weight basis and linear ($r^2 = 0.99$) as fruits matured from class four (whitish-green) to eight (light purple), and similar trends in TBARS with development were evident when expressed on a whole fruit and dry weight basis. Accumulation of these lipid peroxidation products is evidence of increasing oxidative stress during the ripening phase of fruit development.

Peroxidation can affect the concentration and degree of unsaturation of fatty acids in various lipid fractions. For example, activated oxygen can directly deesterify fatty acids from membrane associated lipids (San Filippo et al., 1976; Niehaus, 1987; McKersie et al., 1988). Free polyunsaturated fatty acids (PUFA) in particular serve as substrates for LOX which produces oxy-free radicals and lipid hydroperoxides, the latter decomposing into ethane and TBARS. Hence, oxidative stress can lead to changes in fatty acid concentrations and relative unsaturation ratios in various lipid pools. Changes in concentrations and DBIs of fatty acids in polar lipid (membrane) and free fatty acid fractions were characterized in fruit pericarp with advancing maturity. The concentration of polar lipid fatty acids declined 35% (371 nmol g fresh wt^{-1}) as fruit developed from class four to nine (Table 5-1). This decrease was mainly attributable to a 65% (260 nmol g fresh wt^{-1}) reduction in 18:3 (Fig. 5-2). The concentration of free fatty acids also fell substantially with advancing maturity (Table 5-1, Fig. 5-2). While the DBI of free fatty acids was not significantly affected by fruit development, DBI of polar lipids decreased with maturation (Table 5-1) and this was attributed mostly to the loss of linolenic acid (18:3 fell from 40 to 20 mole percent as fruits developed from class four to nine). The increase in relative saturation of polar lipids may have resulted from a combination of direct peroxidation of membrane-bound fatty acids and their deesterification by lipolytic enzymes, as has been documented for other senescing systems (Borochov et al., 1982; Droillard et al., 1993). Regardless of the mechanism, increases in lipid peroxidation products (ethane, TBARS), in conjunction with a progressive loss of unsaturation of polar lipids, is indicative of increasing oxidative stress during fruit development and ripening.

Membrane-bound and free fatty acids can be directly oxidized by O_2^- and other active oxygen species (Niehaus, 1978; Senaratna et al., 1985). Cells prevent such macromolecular oxidative damage by maintaining a reduced environment through various mechanisms. Under normal (nonstressful) conditions, active oxygen species produced during metabolism are efficiently neutralized by various free-radical scavengers (e.g. ascorbate, α-tocopherol) and/or are catabolized enzymatically. Oxidative stress results from a shift in the equilibrium between free radical production and consumption through these pathways to favor production (Hamilton, 1991; Purvis et al., 1995). For example, SOD activity decreases during senescence in many plant systems resulting in an increase in O_2^- and thus oxidative stress (Dhindsa et al., 1981). Similarly, various environmental stresses (drought, chilling) have been shown to effect an increase in free radicals and lipid hydroperoxides due to reduced activity of CAT (Hertwig et al., 1992), which catabolizes the cytotoxic H_2O_2 resulting from SOD activity. To determine whether the increasing oxidative stress accompanying maturation and ripening of saskatoon fruit was associated with reduced ability to enzymatically catabolize active oxygen species, changes in activities of SOD, CAT, POX, GRase and GTase were characterized as a function of fruit development.

SOD and CAT activities (protein basis) were highest in mature green fruit (class four), but declined 4-fold and 18-fold, respectively, as fruit developed to the fully ripe, class nine (purple) stage (Fig. 5-3). POX activity remained low and constant as fruit developed from class four to eight (dark red stage), then increased 8-fold to maturity class nine. Changes in the specific activities of these enzymes were not simply a consequence of changes in fruit protein content. Protein on a per fruit basis declined 35% from class four to nine and similar trends in the activities of SOD, CAT and POX were apparent on a fruit fresh and dry weight basis (data not shown). These results thus correlated well with the progressive increase in oxidative stress during fruit development, as characterized by the accumulation of ethane and TBARS (Fig. 5-1), decline in fatty acid concentrations, and increase in saturation of polar lipids (Table 5-1, Fig. 5-2). Respiration is a primary source of O_2^- (Cadenas et al., 1977; Forman and Boveris, 1982; Purvis et al., 1995). Due to a substantial increase in respiration of this climacteric fruit (Rogiers and Knowles, 1996), oxy free radical production likely increases over the latter stages of development (maturity classes five to nine). The concomitant decline in SOD and CAT activities would thus contribute to accumulation of O_2^- and H_2O_2, affecting increased oxidative stress during development.

Increases in lipid peroxidation products and saturation of membranes during the latter stages of fruit development could also be mediated through increased LOX activity. LOX catalyzes the oxygenation of free and esterified PUFAs, producing lipid hydroperoxides in the process. Tissue prints of saskatoon fruit showed that LOX was detectable in the pericarp at all stages of development (Fig. 5-4). On a per fruit basis, LOX was most concentrated in immature fruit and declined with advancing maturity. LOX activity however increased 2.5-fold on a protein basis as fruit ripened from class five to nine (Fig. 5-3), and SDS-PAGE and Western blots showed this to be due to a progressive increase in the amount of enzyme per unit protein (Fig. 5-5). The apparent decline in LOX concentration in tissue prints (Fig. 5-4) was no doubt a consequence of the rapid gain in water during development, resulting in dilution of total protein (Rogiers and Knowles, 1997) and thus also the enzyme.

Oxidative stress is often prevented by higher activities of SOD, CAT and POX (Bowler et al., 1992; Purvis et al., 1995), all of which are inducible (Hassan and Scandalios, 1990; Finazzi-Agro et al., 1986). As previously noted, increased activities of these free radical scavenging enzymes prevents the buildup of O_2^- and H_2O_2, enhancing a cell's ability to maintain a reduced environment and thus contain oxidative stress. During plant senescence, activities of SOD, CAT and POX often fall, resulting in a concomitant decline in ability to scavenge free radicals (Dhindsa et al., 1981). Trends in the activities of LOX, SOD and CAT in developing saskatoon fruit were thus consistent with those evident in true senescing systems, even though the fruit were in an active growth stage and had not yet begun to senesce. The increasing oxidative stress that likely results from lower activities of these enzymes is evidently needed to facilitate many of the metabolic changes associated with maturation and ripening of saskatoon fruit.

The glutathione system is also directly involved in maintaining a low redox potential and thus a highly reduced intracellular environment (Tanaka et al., 1994). Glutathione levels increase in plants to counteract oxidation resulting from drought stress (Dhindsa and Matowe, 1981), chilling stress (Walker and McKersie, 1993), or injury by ozone (Guri, 1983) or H_2O_2 (Smith et al., 1984; Smith, 1985). Reduced glutathione (GSH) can react with singlet oxygen, O_2^-, hydroxyl radicals and free lipid hydroperoxides, and may also stabilize membrane structure by removing acyl peroxides formed by lipid peroxidation (Price et al., 1990). As such, GSH is a potent inhibitor of lipid peroxidation and an efficient free-radical scavenger (Haenen, 1989). This tripeptide oxidizes thiol groups with the subsequent formation of the glutathione dimer conjugate GSSG (Leshem et al., 1986) and thus maintains a reduced environment for the cell. Increases in concentrations of

GSH and oxidized glutathione (GSSG) are often indicative of increases in oxidative stress. In fact, glutathione titres have been positively correlated with ability to counteract oxidative stress (May and Leaver, 1993).

Saskatoon fruit responded to the increase in oxidative stress during development by increasing glutathione titres. Both GSH and GSSG concentrations increased about 2-fold as fruit developed from maturity class six to nine (Fig. 5-6). These increases coincided with the climacteric rise in respiration during ripening (Rogiers and Knowles, 1996) and since respiration is a major source of activated oxygen species (Forman and Boveris, 1982; Rich and Bonner, 1978), the respiratory climacteric is likely a significant contributor to the increasing oxidative stress during this phase of development. Moreover, GSSG increased from about 6 to 10% of total glutathione as fruit matured from class six to nine, indicating an increase in cellular oxidative status during ripening (Fig. 5-6 inset). The higher ratio of GSSG to total glutathione in earlier developmental stages likely reflects higher metabolic rates associated with more active growth, rather than oxidative stress.

GTase exhibits peroxidase activity (Ketterer et al., 1990; Bartling et al., 1993) and is responsible for catabolizing lipid hydroperoxides that form during the propagation phase of lipid peroxidation, oxidizing GSH in the process (Haenen, 1989). The GSSG produced from GTase activity is then reduced by GRase, with NADPH as a source of electrons (Carlberg and Mannervik, 1985). Tissue prints of GRase and GTase indicated that these enzymes are easily detectable at all stages of development, uniformly distributed throughout the pericarp, and most concentrated in immature fruit on a per fruit basis (Fig. 5-4). The activities of these enzymes increased only marginally as fruit developed from class four to eight (Fig. 5-6). However, as fruit matured from class eight to nine, GRase and GTase activities increased 5- and 6-fold, respectively. Western blots showed that the increase in activities from maturity class eight to nine were likely due to an increase in the levels of these enzymes per unit protein (Fig. 5-5). In the absence of any notable decline in other proteins during this period, it is evident that saskatoon fruit respond to the progressive increase in oxidative stress during earlier stages of maturation by upregulating the glutathione system during the final stages of ripening.

In summary, ripening of saskatoon fruit was accompanied by a progressive increase in oxidative/peroxidative stress that significantly affected increased saturation of polar lipids. The drop in DBI of this fraction was mostly attributable to a loss of linoleneate, which is a major substrate for LOX. Lipid hydroperoxides from higher LOX activity, in conjunction with a decline in SOD/CAT activities and increased respiration during ripening, likely contributed to an increase in cellular oxidative status as proposed in Fig. 5-7. Increased production of lipid hydroperoxides and other active oxygen species during development eventually induced higher activities of POX, GRase and GTase, but not until the latter stages of ripening. Although speculative, it appears that a decline in free radical scavenging ability and the associated increase in oxidative stress may be requisites for mediating many of the physico-chemical changes that facilitate maturation and ripening of this pome fruit.

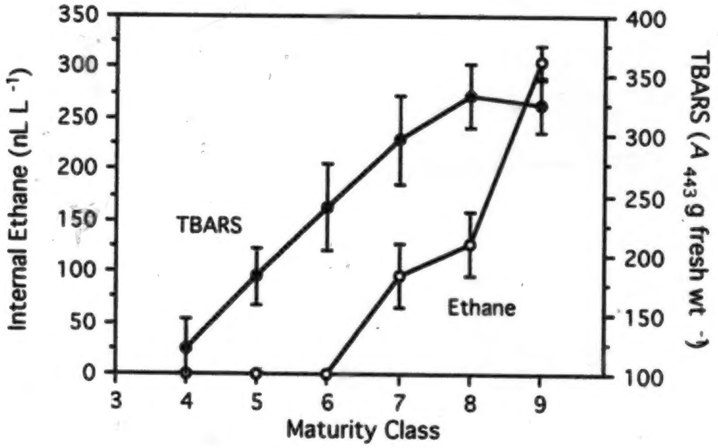


Fig. 5-1. Changes in internal ethane and TBARS of saskatoon fruit with development from the mature green stage (class four) to the fully ripe purple stage (class nine). Maturity classes are as defined in Rogiers and Knowles (1997) (see Materials and Methods and Introduction for further description). Error bars represent ±SE.

Table 5-1. *Changes in free and polar lipid fatty acids with advancing maturity of saskatoon fruit.*

Lipid Fraction	Units	Fruit Maturity Class[a] 4	7	9	Linear Trend
Polar Lipids	nmol g fresh wt^{-1}	1062 ± 196	964 ± 43	691 ± 135	0.05[c]
	DBI[b]	6.50 ± 0.14	5.97 ± 0.24	5.48 ± 0.40	0.05
Free Fatty Acids	nmol g fresh wt^{-1}	49.8 ± 6.6	43.7 ± 11.0	20.9 ± 4.8	0.05
	DBI	1.68 ± 0.40	2.54 ± 0.56	1.15 ± 0.32	NS

[a] Fruit maturity classes are as indicated in Fig. 5-1 (classes 4, 7 and 9 are mature green, dark red and purple stages, respectively, as defined in Rogiers and Knowles (1997)).
[b] Double bond index (DBI) calculated based on mole percent of fatty acids in fraction.
[c] Significance levels for indicated trends with maturity class.

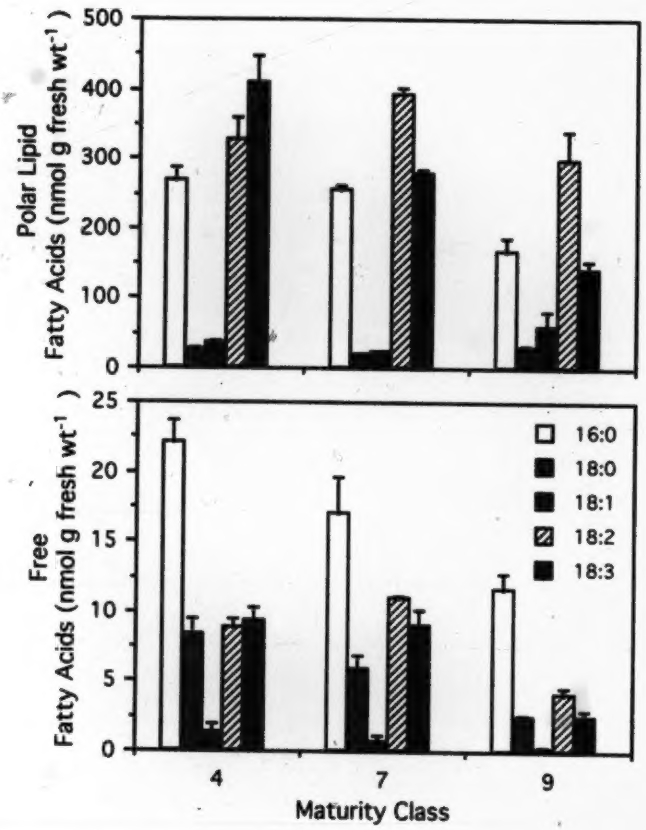


Fig. 5-2. Changes in concentrations of polar lipid and free fatty acids of saskatoon fruit with development from the mature green stage to the fully ripe purple stage. Maturity classes are as defined in Fig. 5-1. Error bars represent ±SE.

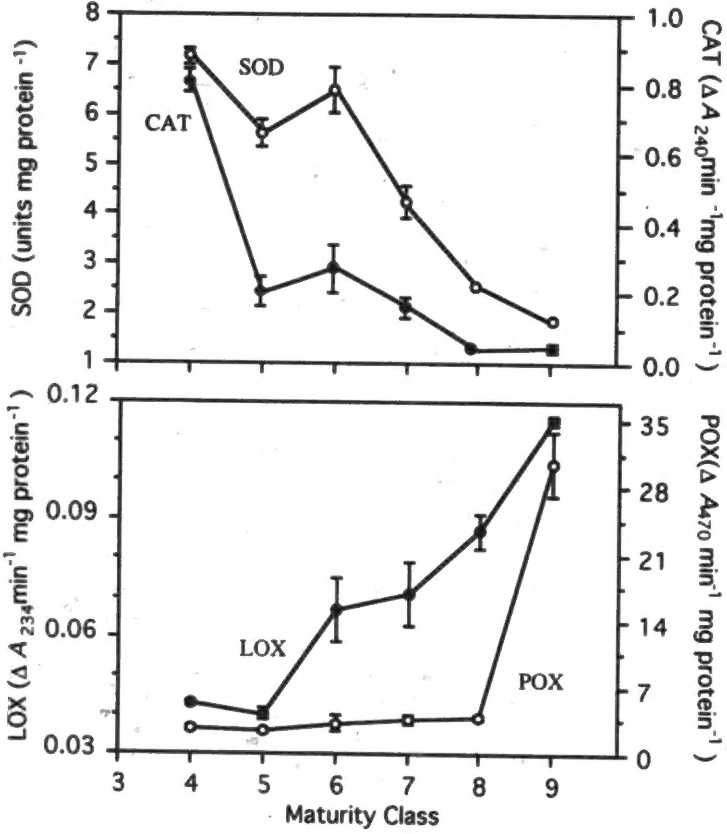


Fig. 5-3. Changes in SOD, CAT, POX and LOX activities with development of saskatoon fruit from the mature green stage to the fully ripe purple stage. Maturity classes are as defined in Fig. 5-1. Error bars represent ±SE.

Fig. 5-4. Tissue prints of LOX, GRase and GTase from transverse sections of developing saskatoon fruit. Prints were incubated with anti-potato LOX, anti-spinach leaf GRase, and anti-*Hyoscyamus muticus* GTase. Maturity classes are as defined in Fig. 5-1 and in Rogiers and Knowles (1997).

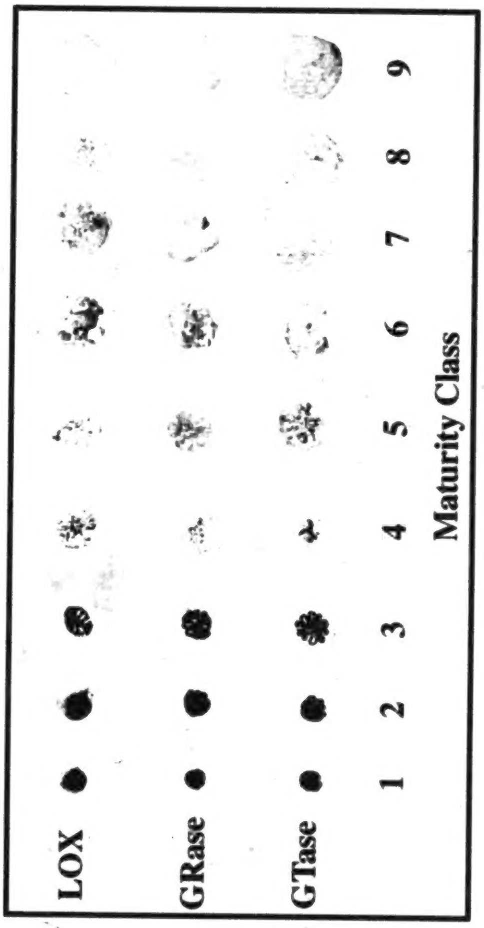
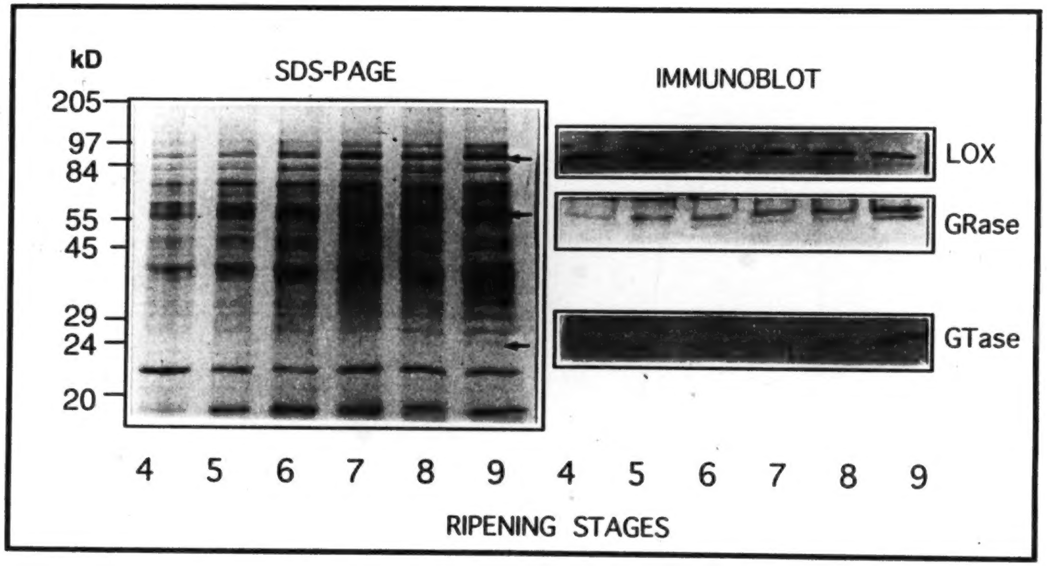

LOX
GRase
GTase
1
2
3
4
5
6
7
8
9
Maturity Class

Fig. 5-5. SDS-PAGE of soluble proteins and Western analysis of LOX, GRase and GTase from developing saskatoon fruit (80 mg protein/lane). Maturity classes are as defined in Fig. 5-1. The 92, 85 and 25 kD polypeptides cross-reacted with anti-potato LOX, anti-spinach leaf GRase and anti-*Hyocyamus muticus* GTase, respectively.


kD
SDS-PAGE
IMMUNOBLOT
205
97
84
55
45
29
24
20
LOX
GRase
GTase
4
5
6
7
8
9
4
5
6
7
8
9
RIPENING STAGES

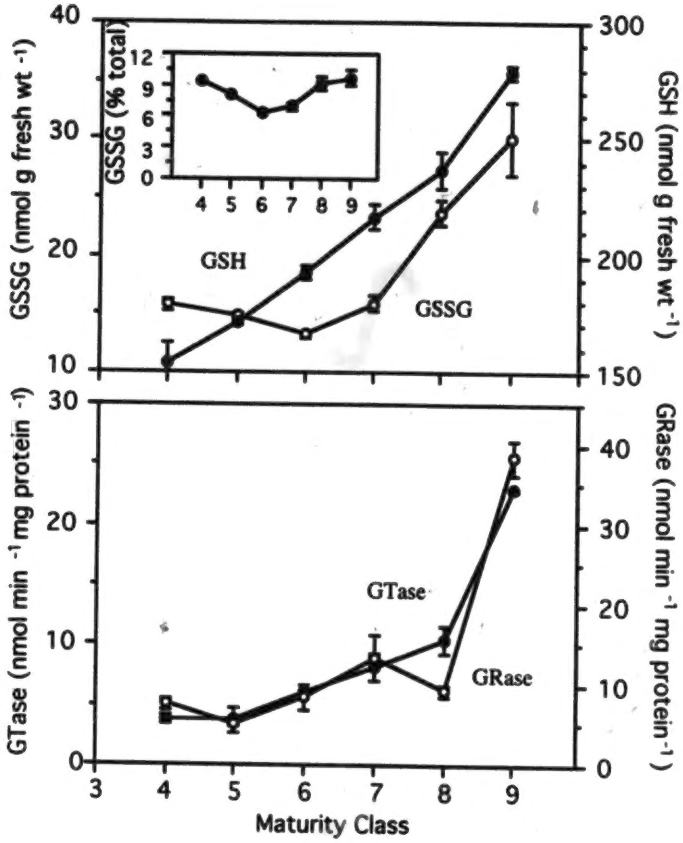
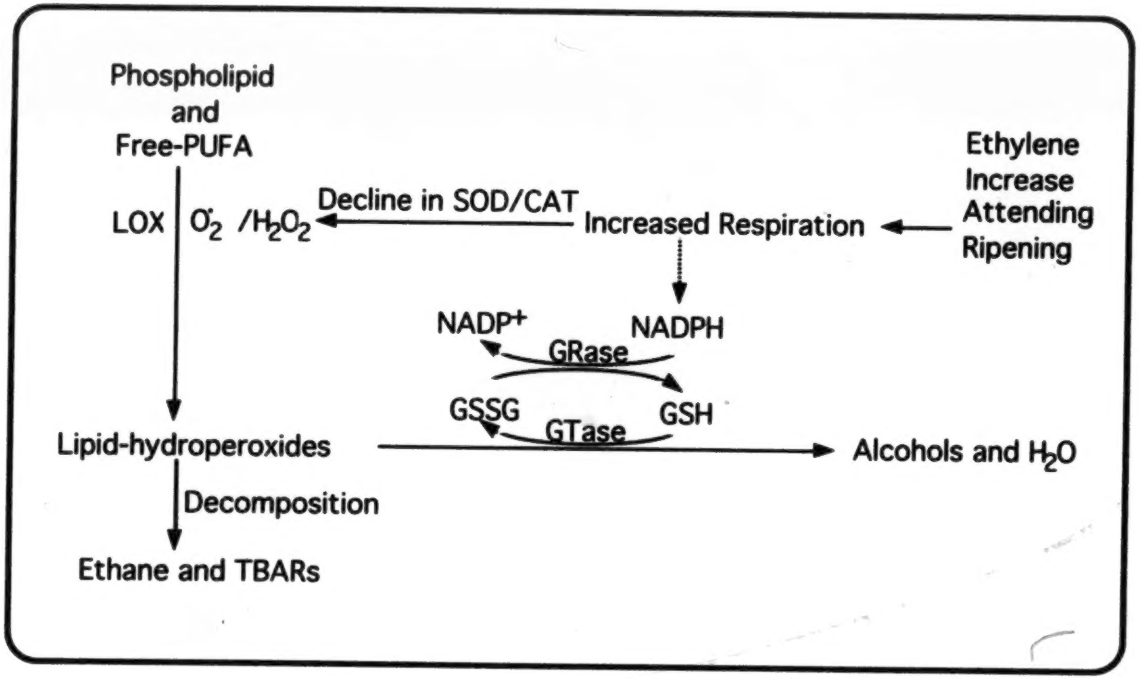


Fig. 5-6. Changes in glutathione concentrations and activities of GRase and GTase with development of saskatoon fruit from the mature green stage to the fully ripe purple stage. Maturity classes are as defined in Fig. 5-1. Inset shows change in GSSG as a percentage of total glutathione. GRase and GTase activities are nmol of NADPH oxidized and S-2,4-dinitrophenyl-GSH formed per minute expressed on a protein basis, respectively. Error bars represent ±SE.

Fig. 5-7. A summary of the various systems contributing to (or responding to) oxidative stress during ripening of saskatoon fruit. From maturity classes four to nine, respiration and ethylene production rates increased for this climacteric fruit (Rogiers and Knowles, 1996). The increased superoxide production from respiration, in conjunction with decreased activities of SOD and CAT, likely resulted in a greater potential for oxidative damage. Superoxide and hydrogen peroxide can directly oxidize/peroxidize free or esterified PUFA, yielding lipid hydroperoxides. Similarly, production of lipid hydroperoxides are a direct consequence of the increased LOX activity associated with ripening. Increased lipid peroxidation during ripening was evidenced by a decline in DBI of polar lipids, and buildup in ethane and oxidative stress and, as a consequence, glutathione titres increased. The increased NADPH consumed by GRase represents an additional energy sink to which respiration would respond.


Phospholipid and Free-PUFA
LOX
$O_2^{\cdot}$ /H_2O_2
Decline in SOD/CAT
Increased Respiration
Ethylene Increase Attending Ripening
NADP+
NADPH
GRase
GSSG
GSH
GTase
Lipid-hydroperoxides
Alcohols and H_2O
Decomposition
Ethane and TBARs

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Chapter VI

Effects Of Storage Temperature and Atmosphere On Saskatoon (*Amelanchier alnifolia* Nutt.) Fruit Quality, Respiration and Ethylene Production

Abstract

Low temperature and controlled atmospheres (CA) were tested for their effectiveness at extending the storage life of saskatoon fruit. Quality of ripe maturity class nine 'Smoky' and 'Pembina' fruit was assessed over 40 days of storage at 0.5 or 4.0°C in humidified (95%) air. Fungal colonization, fruit softening, fresh weight loss, and anthocyanin degradation were substantially lower in fruit stored at 0.5°C for 40 days, as compared with those stored at 4.0°C. Soluble solids content of cv. Pembina fruit was not affected by temperature over the 40 d period; however, 'Smoky' fruit maintained higher soluble solids when stored at 0.5°C than at 4.0°C. Fruit (cvs. Smoky and Northline) of maturity classes seven, eight, and nine were stored in 2%, 10%, or 21% O_2 at 4.0°C (95% RH), and respiration and ethylene evolution were monitored over 10 days. The rate of ethylene production by fruit of both cultivars decreased linearly with advancing maturity class. Ethylene production rate by fruit of all three maturity classes also decreased with declining O_2 concentration. Furthermore, the increase in ethylene production rate of maturity class nine (fully ripe) fruit with increasing O_2 concentration was less than that from maturity classes seven and eight fruit. Fruit respiration was lowest in the 2% O_2 atmosphere and highest in the 21% O_2 atmosphere for 'Smoky' fruit; however, respiration rate of 'Northline' fruit was lower only in the 2% O_2 atmosphere. Collectively, our results show that storage at 0.5°C was more effective than 4.0°C at maintaining fresh quality of saskatoon fruit. Low O_2 atmosphere (2%) effectively slowed ethylene production and fruit respiration and thus may be a beneficial adjunct to low temperature storage.

Introduction

Amelanchier alnifolia Nutt. produces a small fruit with a sweet almondy flavor. Fruit are approximately 1.5 cm in diameter and purple when ripe. The fruit is a miniature pome that developes from a gynoecial hypanthium, and is born on shrubs in raceme-like, determinate infructescences containing 8-12 fruit (Steeves and Steeves, 1990; Steeves et al., 1991). In recent years the saskatoon fruit industry has rapidly expanded and currently there are about 1100 ha in production across the prairies of Canada (Hausher, 1996 personal communication). The demand for saskatoon fruit exceeds supply (St. Pierre, 1992) with the fruit either consumed fresh, mainly from 'U-pick' operations, or machine-harvested and processed into value-added products. Although there is current demand for saskatoons as a fresh product at the retail level, lack of adequate transport, storage, and packaging systems for maintaining quality of this highly perishable fruit have impeded development of a fresh market industry.

Refrigeration is currently the most effective method for retarding postharvest deterioration of fruits and vegetables (Knee et al., 1983). Significant improvements in storage life are achieved by small reductions in temperature at the lower end of the nonstressful temperature range for a particular fruit (Wills et al., 1989). Low temperature slows the metabolism of produce and limits the rate of microbial growth in storage. In climacteric fruits, low temperature also decreases ethylene production and the rate at which tissues respond to ethylene (Kader et al., 1989). Ethylene mediates ripening of saskatoon fruit, as evidenced by increased ethylene production and content with advancing maturity and the ability of exogenous ACC to stimulate ethylene biosynthesis and ripening of preclimacteric fruit (Rogiers et al., 1997). Therefore, low temperature storage may be effective in preserving the fresh quality of saskatoon fruit by attenuating pathological deterioration, ripening and senescence. No information is available on the efficacy of low temperature storage in prolonging the postharvest life of saskatoon fruit. A main objective of this study was to compare the effects of cold storage on slowing the deterioration of fruit over a prolonged storage period.

Similar to refrigeration, low O_2 storage is effective at slowing ripening of various climacteric fruit, through lowering respiration rates, ethylene production, and sensitivity of the tissue to ethylene. Ethylene production by apple, pear, and plum fruit was reduced in low O_2 atmospheres (Ke et al., 1991). Low O_2 has been especially successful for storage of apples which can maintain quality for up to a year in 2% O_2 (Abeles et al., 1992). Apples and saskatoons are taxonomically related, have similar developmental origins, and are both climacteric. Hence, as with apples, low O_2 storage may be effective for prolonging the postharvest life of saskatoon fruit. The effects of low O_2 on rates of respiration and ethylene production of saskatoon fruit in refrigerated storage were thus characterized.

Materials and Methods

Fruit Source

Saskatoon fruit (*Amelanchier alnifolia* Nutt.) were hand harvested from an orchard in Spruce Grove, Alberta. The fruit were transported on ice, cleaned of debris and sorted into maturity classes based on the maturity index devised by Rogiers and Knowles (1997). Fruit of maturity class seven were dark red, maturity class eight fruit were dark red with purple hues, and maturity class nine fruit were purple and considered optimum for consumption. Maturity class nine fruit of the cvs. Pembina and Smoky were used for the temperature study. Maturity classes seven, eight, and nine fruit of the cvs. Smoky and Northline were used for the controlled atmosphere study. 'Northline' and 'Parkhill' (*Amelanchier sanguina* Nutt.) fruit of maturity class nine were used in a study to show that 2% O_2 (the lowest O_2 treatment) was above the pasteur point of saskatoons. Fruit were placed in the appropriate storages (see below) within 5 hours of harvest.

Low Temperature Study

Fruit (150 g) were packaged in nylon mesh (0.3 mm^2) sachets and placed into 10 L plexiglass chambers inside walk-in coolers at 0.5 and 4°C in the dark (Fig. 6-1). The fruit were exposed to a continuous flow (100 mL min^{-1}) of humidified air for up to 40 days. There were three replicate samples per harvest date for each cultivar (Pembina and Smoky) at each temperature, and harvest order was determined at the beginning of the study. Fruit were harvested from the chambers at 7 to 14 day intervals throughout the study. Fruit firmness, weight loss and fungal decay were assessed immediately, and a sample of fruit from each treament was then frozen at -30°C for subsequent analysis of soluble solids, anthocyanins, and pH.

Fungal growth on fruit was assessed visually, with fruit rated as 0%, 1-30%, 31-60%, or 61-100% colonized. Analysis was based on the midpoint of each category. Fruit firmness was measured with an Instron Universal Testing Machine, model 4201 (Instron Corp., Canton, MA) equipped with a 50 kg load cell. The downspeed of the crosshead was 150 mm min^{-1}. A fruit sample (15 g) was placed in a Kramer Shear Compression Cell (Instron Corp.), adjusted by the insertion of two metal plates so that the bottom area was 14 cm^2 (Gill et al., 1979). The return gauge was set so that the compression cycle terminated as the blades emerged from the bottom of the test cell. Fruit firmness was expressed as the force required to penetrate the sample in kg g^{-1} fresh weight. To determine soluble solids, frozen fruit (5 g) were macerated with a polytron

(Brinkmann, Rexdale, Ont.) at room temperature and extracts were passed through miracloth. The filtrates were centrifuged for 20 min (1640 g) and an ATAGO hand refractometer, model 500 (ATAGO Co., Ltd., Tokyo, Japan) was used to quantify the refractive index of each supernatant. The refractometer was calibrated with 0% and 10% sucrose solutions and refractive index values were adjusted to 20°C. For pH analysis, frozen fruit (10 g) were extracted with 25 mL of water using a polytron (1 min). The sample was centrifuged at 14,500 g for 20 min (4°C) and pH of the supernatant was determined by the electrode method (AOAC, 1995).

Anthocyanins were extracted from 5 g of frozen fruit with 10 mL of 95% (v/v) ethanol:1.5 M HCl (85:15 v/v) at 4°C using a polytron. Extracts were centrifuged at 14,500 g for 15 min (4°C) and pellets were washed twice with 5 mL of the extraction medium. The supernatants were combined and diluted to 25 mL with extraction medium. Anthocyanins were assayed by the pH differential method of Fuleki and Francis (1968). Extract (100 µL) was added to 2.4 mL of 0.2 N KCl:0.2 N HCl (25:67) buffer (pH 1.0). In addition, 200 µL of extract was combined with 800 µL of 1.0 N sodium acetate:1.0 N HCl:H_2O (100:60:90) buffer (pH 4.5). The samples were then equilibrated in the dark for 2 h and A_{510} was recorded. The absorbance at pH 1.0 was subtracted from that at pH 4.5 and total anthocyanins were calculated using the extinction coefficient for cyanidin-3-galactoside ($\varepsilon^{1\%}$ 510 nm=765), the predominant anthocyanin of saskatoon fruit (Mazza, 1986).

Controlled Atmosphere Storage Studies

To characterize the effect of O_2 concentration on fruit respiration rate, and to identify suitable O_2 levels for subsequent storage studies, 20 g samples (3 replicates) of cv. Northline fruit (maturity class nine) were placed inside 80 mL glass tubes (2.5 cm i.d. x 22.0 cm), the ends of which were closed with rubber septa. A humidified continuous flow (15 mL min^{-1}) of 1.7, 4.0, 5.8. 9.5 and 23% O_2 (balance N_2) was established through 18 gauge hypodermic needles piercing each septum. After 24 h at 23°C, 1 mL of the outflow from each tube was analyzed for CO_2 by GC (see below) and respiration rates (fresh wt basis) were plotted against O_2 concentration.

While this study characterized the overall respiratory response of fruit to declining O_2 concentration, it did not indicate whether anaerobic respiration had been invoked at the lower levels of O_2 . It was important to determine that the concentration of O_2 selected for the storage study (2%) was above the pasteur point for saskatoon fruit. Hence, an additional study, utilizing a similar flow-through system, profiled the respiratory response of individual fruit (2 replicates) to air (21% O_2) or 2% O_2 (balance N_2) continuously for 80 h at 23°C. Fruit respiration was determined by analyzing CO_2 in the the outflow from each chamber at 30 min intervals with an LI-6262 CO_2/H_2O infra-red gas analyzer (LI-COR, Inc., Lincoln, Nebraska). Since a pasteur effect on respiration was not observed during the first 40 h at 2% O_2, the O_2 was reduced to zero and respiration was monitored for another 40 h. A pasteur effect was subsequently observed.

For the low O_2 storage study, 150 g samples (3 replicates) of 'Smoky' and 'Northline' fruit of maturity classes seven, eight, and nine were placed inside 500 mL glass cylinders (4.7 cm i.d. x 30.0 cm), the ends of which were closed with rubber stoppers bored to provide inlet and outlet ports. The cylinders were placed inside a walk-in cooler at 4°C and humidified atmospheres of either 2%, 10%, or 21% O_2 (balance N_2) were established at 30 mL min^{-1} through the cylinders. Samples (1 mL) of the outflow from each chamber were analyzed for ethylene and CO_2 daily over a 10 day storage interval. Ethylene was quantified on a Photovac 10A10 Portable Photoionization GC (Photovac Inc., Thornhill, Ontario, Canada) equipped with an 8' x 1/8" teflon column packed with Porasil B (100-150 mesh). The flow rate of the carrier gas (air) was 15 mL min^{-1}. Carbon dioxide was analyzed on a Hewlett-Packard 5890A GC, equipped with a 2.4 m stainless steel column (3.2 mm o.d.) packed with HayeSep T (Hewlett Packard) and a thermal conductivity detector. The carrier gas (He) flow rate was 30 mL min^{-1} and the column was isothermal at 100°C. Injection and detector port temperatures were 140°C.

Results and Discussion

Storage of maturity class nine saskatoon fruit at 0.5°C was more effective than 4°C at slowing pathological deterioration (Fig. 6-2). There were no signs of fungal colonization over the first 22 days of storage at either temperature. After 40 days at 0.5°C however, less than 30% of fruit showed signs of colonization, while at 4.0°C, more than 80% of fruit were colonized. The predominant pathogen was grey mold (*Botrytis cinerea* Pers.), and infection of cv. Pembina fruit was more rapid than that of 'Smoky' fruit at 4°C. At 0.5°C the rates of fungal colonization of fruit of the two cultivars were equal.

When averaged over cultivar, fruit firmness declined only 8% over 40 days of storage at 0.5°C, compared with 35% when stored at 4°C (Fig. 6-3A). Furthermore, fruit stored at the lower temperature maintained firmness significantly longer (at least 5 days) than those stored at the higher temperature. Firmness of 'Pembina' fruit was higher than that of 'Smoky' fruit at harvest; however, 'Pembina' fruit began to soften 5 days earlier than 'Smoky' fruit and firmness was comparable for fruit of the two cultivars after 33 days of storage (averaged over temperature) (Fig. 6-3B). When averaged over storage period, 'Pembina' and 'Smoky' fruit were 15% and 11% firmer, respectively, when stored at 0.5°C than at 4°C (Fig. 6-3B inset).

Fruit soluble solids content declined by only 7% over the 40 day storage period and the rate of decline was not affected by cultivar or storage temperature (Fig. 6-4). On average, 'Smoky' fruit contained 11.3% more soluble solids than 'Pembina' fruit; however, cultivar interacted with storage temperature to affect soluble solids content. Fruit of cv. Smoky maintained higher soluble solids when stored at 0.5°C than at 4.0°C, while that of Pembina fruit was unaffected by storage temperature.

Fruit weight loss and anthocyanin degradation were less extensive during storage at 0.5°C than at 4°C (Fig. 6-5). Cultivar did not interact with time or storage temperature to affect percent weight loss of fruit. Weight loss was thus equal for fruit of the two cultivars at each temperature over the 40 day storage interval. Fresh weight loss was less than 1.5% at both temperatures over the initial 28 days of storage. From 28 to 40 days, weight loss increased to 4% in fruit stored at 4°C, but remained less than 2% in fruit stored at 0.5°C. Likewise, loss of anthocyanins from fruit stored at 4°C was substantially greater than from fruit stored at 0.5°C over the 40 day interval (Fig. 6-5) and rates of degradation were equal for the two cultivars.

Changes in fruit pH were characterized by a significant 3-way interaction among cultivar, temperature and time (Fig. 6-6). The least change in pH over the 40 day interval was evident in 'Smoky' fruit stored at 0.5°C. At 4°C, pH of 'Smoky' fruit increased from 0 to 20 days of storage, then fell about 17% (about 0.69 pH units) through the remaining 20 days. In contrast, the pH of 'Pembina' fruit remained relatively constant over the initial 28 days of storage at 0.5°C and then decreased. The pH of 'Pembina' fruit declined earlier at 4°C than at 0.5°C, but was about equal at the two temperatures after 40 days of storage.

Collectively, our results indicate that physiological and pathological deterioration of saskatoon fruit are attenuated by storage at 0.5°C, as compared with 4°C, resulting in better quality and a potentially longer postharvest life at the lower temperature. Interactions of cultivar with time and temperature to effect various quality parameters suggests that some cultivars may be better suited to storage than others. This is not surprising since a genetic basis for storability is prevalent among many fruits (Kader et al., 1989). Since this study characterized changes in only a few of the many physicochemical parameters dictating fruit quality, definitive conclusions on the longest acceptable storage life of fruit of these cultivars are not possible.

Avoiding anaerobic metabolism in CA storages is critical to maintaining fruit quality. Anaerobic respiration can be induced by low O_2 atmospheres and results in off-flavor development due to the increased catabolism of carbohydrate to acetaldehyde and ethanol (Kader, 1987). Knowing the O_2 concentration at which anaerobic respiration is invoked (extinction point) is a prerequisite to defining the lower limit for O_2 in CA storages (Kader, 1987). The O_2 extinction point can be determined by following the drop in CO_2 production rate of fruit as a function of decreasing O_2 concentration. When O_2 declines below the extinction point, CO_2 production rate will increase due to a switch from oxidative catabolism to decarboxylation of pyruvate, which yields acetaldehyde and ethanol (i.e. from aerobic to anaerobic respiration). The resulting increase in CO_2 is known as the pasteur effect (Turner, 1951).

The postharvest respiratory response (CO_2 production) of maturity class nine saskatoon fruit to declining O_2 concentration is shown in Fig. 6-7. After 24 hours of acclimation to 5.8% and 1.7% O_2 at 23°C, respiration had dropped by only 10% and 31%, respectively, when compared with that of fruit under 23% O_2. At 0% O_2, respiration was about 40% of that evident at 23% O_2. Curiously, a pasteur effect was not detected in this particular study. It was expected that CO_2 production by fruit at 0% O_2 would exceed that at 1.7% O_2, due to fermentation of the fruit under complete anoxia. However, it is likely that a significant amount of the CO_2 produced by fruit at 0% O_2 was indeed from fermentation, and profiling respiration over a range of O_2 concentrations between 1.7% and 0% may be necessary to resolve the pasteur effect. The uncertainty of the respiratory response between 1.7% and 0% O_2 is indicated by the dashed line (Fig. 6-7).

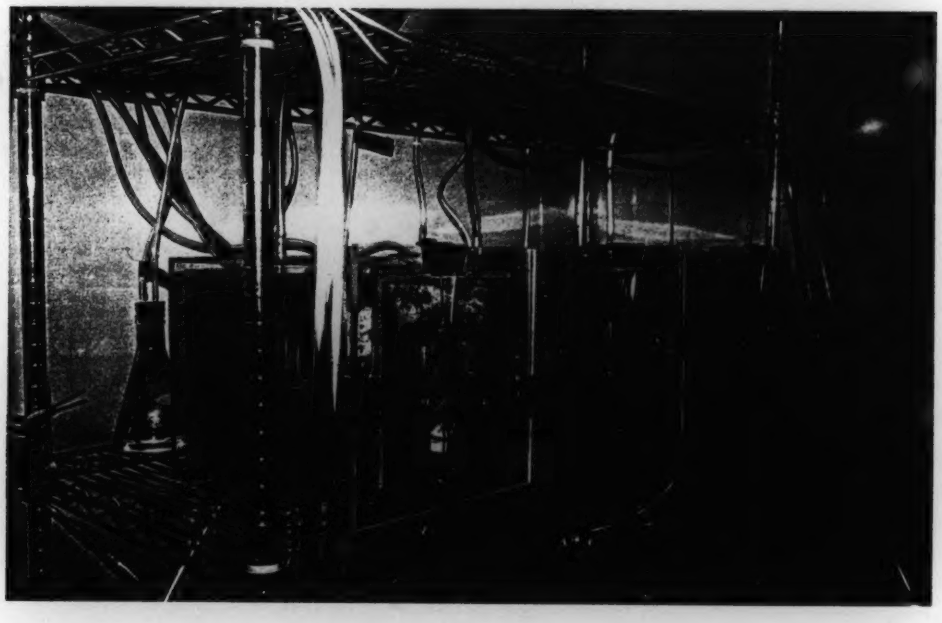
To determine if anaerobic respiration could be induced at 2% O_2, fruit of maturity class nine were placed in a flow-through chamber under 2% or 21% O_2 immediately after harvest and respiration (CO_2 production) was monitored continuously for 80 hours at 23°C. Respiration rate under 2% O_2 declined from 93 μL CO_2 g fwt^{-1} h^{-1} at zero time to 29 μL CO_2 g fwt^{-1} h^{-1} at about 34 h, where it remained constant over the next 8 h (Fig. 6-8). No increase in CO_2 production rate, which would indicate a switch to anaerobic respiration, was evident during the initial 42 h of acclimation to 2% O_2. At 42 h (arrow in Fig. 6-8), the 2% O_2-treated fruit were exposed to complete anoxia, resulting in a 50% drop in CO_2 production rate to 15 μL g fwt^{-1} h^{-1} within 3 h. Respiration rate then increased about 67% over the next 6 h, likely reflecting the onset of anaerobic respiration. Hence, at 23°C, 2% O_2 was above the extinction point for aerobic respiration and a pasteur effect was not observed. As temperature is lowered (e.g. to 4°C as in our CA studies), the O_2 required by fruit and the O_2 extinction point for aerobic respiration are reduced (Kader et al., 1989), therefore anaerobic respiration of saskatoon fruit stored in 2% O_2 at 4°C will be minimal.

The effects of cultivar, time, fruit maturity class and O_2 concentration on ethylene production by fruit were evaluated over a 10 day storage interval at 4°C. There were no physiologically relevant changes in ethylene production over time during storage; however, the rate of ethylene production by 'Northline' fruit was about 2-fold greater than that from 'Smoky' fruit over the storage interval (data not shown). Since cultivar did not interact with any other treatments, data were averaged over time and cultivar to characterize the effects of maturity class and O_2 concentration on ethylene production (Fig. 6-9). Fruit ethylene production was lowest when stored at 2% O_2 and highest at 21% O_2. Ethylene production also declined with advancing fruit maturity class at each O_2 concentration. Moreover, fruit maturity class interacted with O_2 concentration to affect ethylene production. The decrease in ethylene production with advancing maturity class was linear at 10 and 21% O_2, but maturity classes eight and nine fruit had equal rates of ethylene production when stored at 2% O_2. Storage of saskatoon fruit at low temperature did not stimulate ethylene synthesis, as has been shown for various cultivars of pear (Looney, 1972; Sfakiotakis and Dilley, 1974) and apple (Knee et al., 1983).

While changes in fruit respiration rates over time during the 10 d storage period at 4°C were small and insignificant, rates were affected by an interaction between cultivar and O_2 concentration (Fig. 6-10). For fruit of both cultivars, respiration rates were substantially lower when stored at 2% O_2 than at either 10% or 21% O_2. Respiration rates were equal for 'Smoky' and 'Northline' fruit at 21% O_2, but 'Smoky' fruit had a significantly higher respiration rate than 'Northline' fruit at 2% O_2.

Unlike many other climacteric fruits, harvesting and storage of preclimacteric or partially ripened saskatoons is not an option, due to the substantial increase in fruit size that occurs concomitant with ripening (Rogiers and Knowles, 1997; McGarry et al., 1997). Handling and storage systems must thus be effective at slowing deterioration of fully mature, class nine fruit so that fruit size is not sacrificed. Storage at 0.5°C was more effective than 4°C at inhibiting fruit softening, loss in fresh weight and soluble solids, anthocyanin degradation, and fungal colonization of maturity class nine fruit. Reduced O_2 concentration also has the potential of extending storage life, through its ability to depress respiration and ethylene production rates. Oxygen concentration can be lowered to at least 2% in cold storage without invoking anaerobic respiration. Further research on the efficacy of CA storage (both low O_2 and high CO_2) at delaying the postharvest deterioration of saskatoon fruit during longer term storage at 0.5 °C is warranted.

Fig. 6-1. Plexiglass chambers used to evaluate storability of saskatoon fruit at 0.5 and 4.0°C. Fully mature class nine fruit were packaged in nylon mesh bags (150 g each) and placed in the chambers. The chambers were flushed continuously with humidified air (100 mL min^{-1}) during the 40 day storage period. Humidification of the atmosphere in each chamber was accomplished by passing the inflow air through a diffusion stone submerged in distilled water.



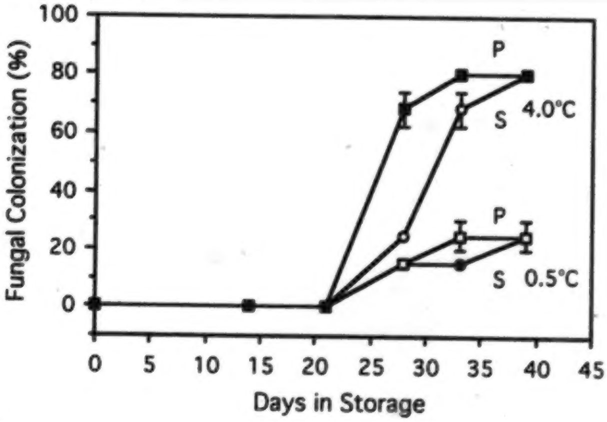


Fig. 6-2. Changes in fungal colonization of maturity class nine 'Pembina' (P) and 'Smoky' (S) saskatoon fruit during storage at 0.5°C or 4.0°C. The fruit were stored as described in Fig. 6-1. Fruit (150 g samples) were ranked subjectively into any one of four categories; 0%, 1-30%, 31-60%, and 61-100%, based on a visual assessment of fungal colonization. Data analysis was based on the midpoint of each category. The predominant fungal pathogen was grey mold (*Botrytis cinerea* Pers.). Except for the cultivar x temperature interaction which was not significant, all other main effects and interactions were significant ($P < 0.05$).

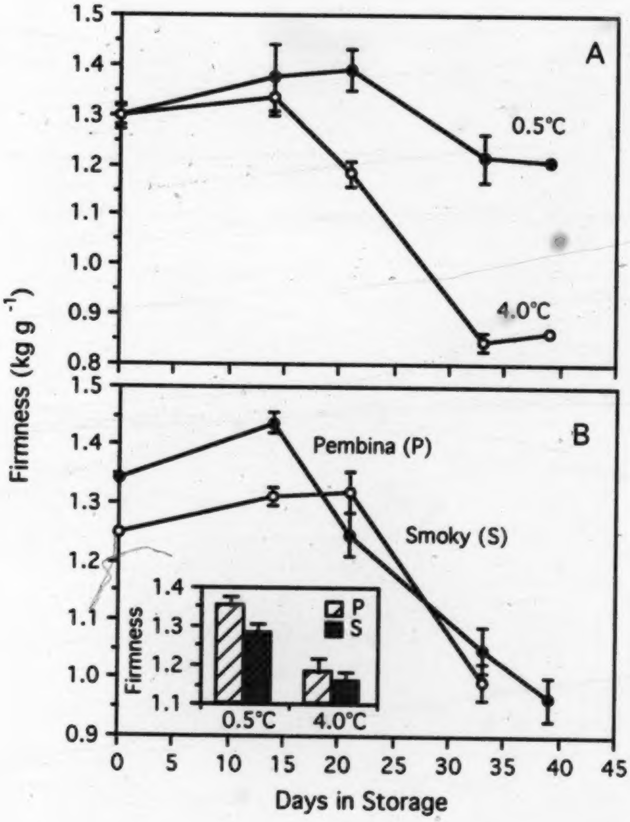


Fig. 6-3. Changes in firmness of maturity class nine saskatoon fruit over 40 days of storage, as affected by temperature (A) and cultivar (B). Fruit were stored as described in Fig. 6-1. All of the two-way interactions were significant ($P<0.05$). The cultivar x temperature x time interaction was not significant.

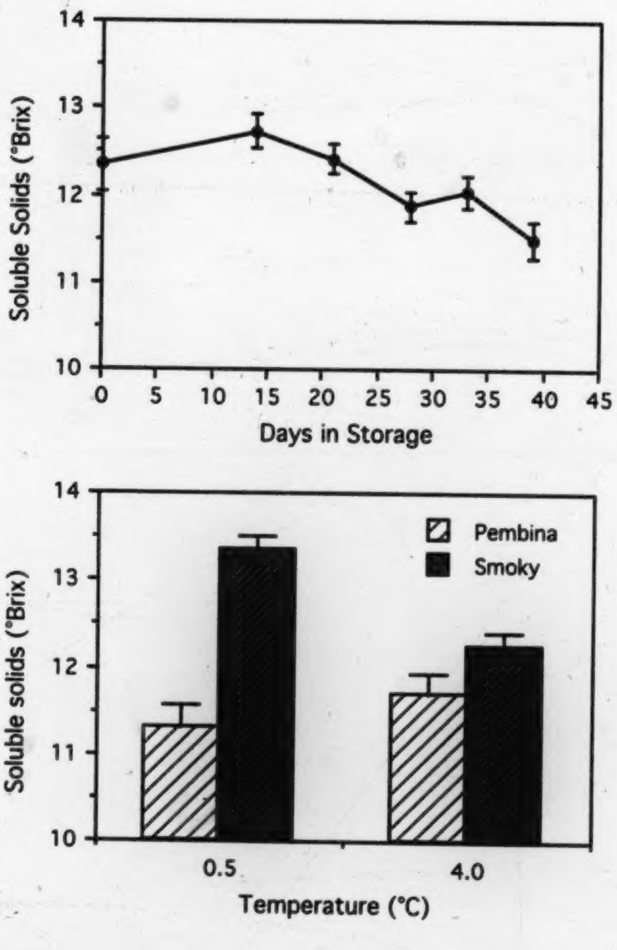


Fig. 6-4. Changes in soluble solids content of maturity class nine saskatoon fruit over 40 days of storage, as affected by cultivar and temperature. Fruit were stored as described in Fig. 6-1. The main effects of cultivar and time and the cultivar x temperature interaction were the only significant ($P<0.05$) treatment effects.

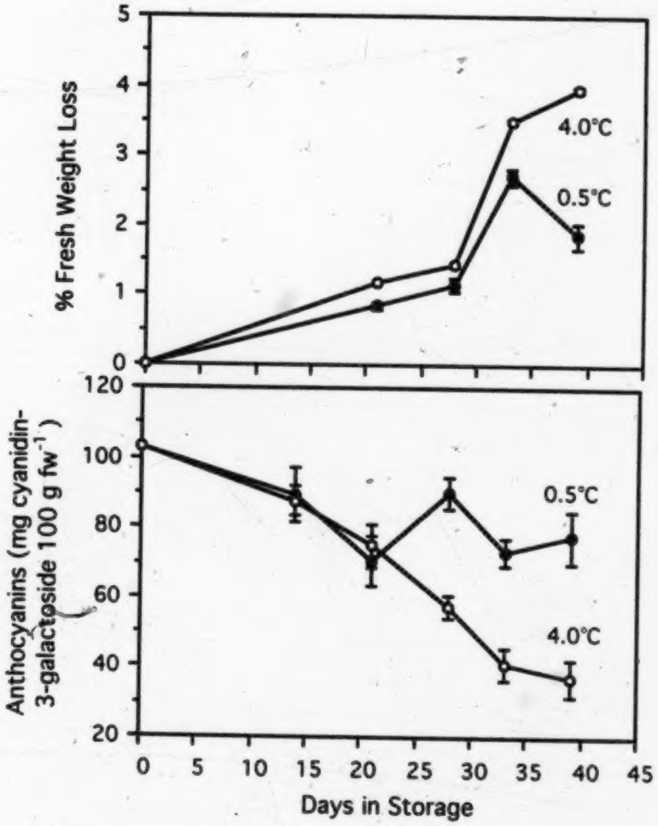


Fig. 6-5. Percent fresh weight loss and changes in anthocyanin concentrations of maturity class nine saskatoon fruit held at 0.5°C or 4.0°C over 40 days of storage. Fruit were stored as described in Fig. 6-1. The interaction of temperature x time was significant ($P<0.05$) for both variables.

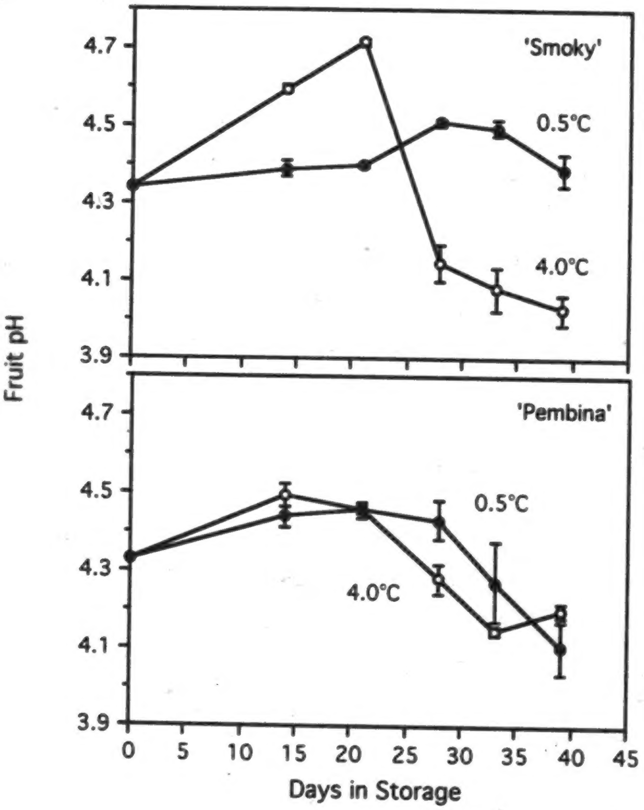


Fig. 6-6. Changes in pH of maturity class nine saskatoon fruit over a 40 day period, as affected by storage temperature and cultivar. Fruit were stored as described in Fig. 6-1. The temperature x time and cultivar x temperature x time interactions were significant ($P<0.05$). All other interactions were not significant.

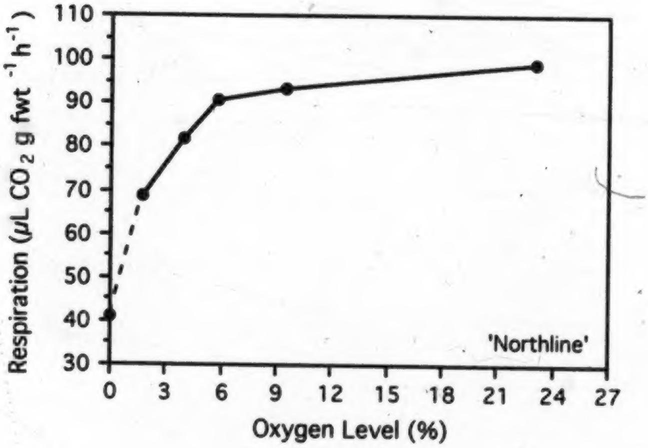


Fig. 6-7. Effect of O_2 concentration on the respiration rate of maturity class nine saskatoon fruit. Fruit were exposed to a constant humidified flow (15 mL min^{-1}) of O_2 (balance N_2) for 24 h at 23°C, whereupon CO_2 was quantified by GC. Data are the means of 3 replicate, 20 g samples of fruit ± standard errors (which are eclipsed by the symbols).

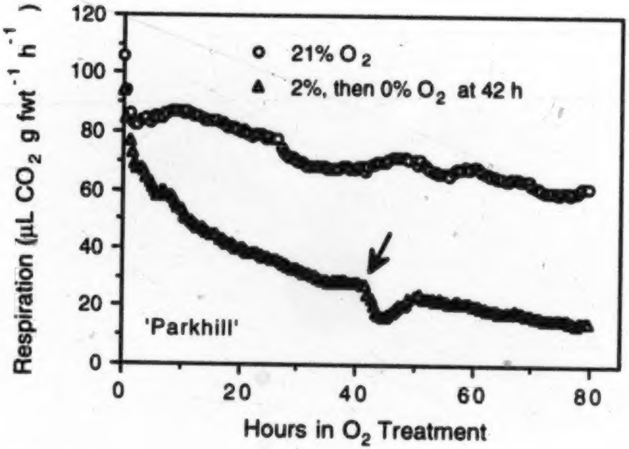


Fig. 6-8. Effect of O_2 concentrations on the respiration rates of maturity class nine saskatoon fruit. Individual fruit were exposed to a constant, humidified flow (15 mL min^{-1}) of air (21% O_2) or 2% O_2 (balance N_2) at 23°C, and CO_2 in the outflow from each chamber was quantified every 30 min for 80 h. At 42 h, the 2% O_2 treatment was changed (arrow) to complete anoxia (100% N_2). Data are means of 2 replicates.

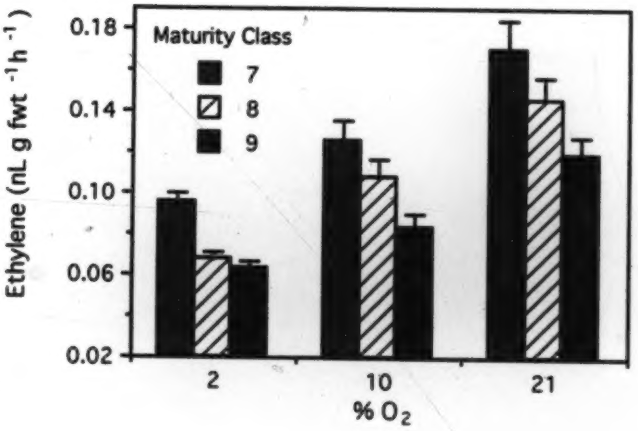


Fig. 6-9. Effects of O_2 atmospheres (balance N_2) and fruit maturity classes on ethylene production rates of saskatoon fruit in storage at 4°C. Fruit were ventilated (30 mL min^{-1}) constantly with the appropriate O_2 atmosphere during the 10-day storage interval. Data were averaged over two cultivars ('Northline' and 'Smoky') and storage time to show the O_2 concentration x maturity class interaction ($P<0.05$). Except for the main effect of cultivar, all other interactions were not significant. Maturity class seven, eight, and nine fruit were dark red, partly purple and purple, respectively (see Rogiers and Knowles, 1997).

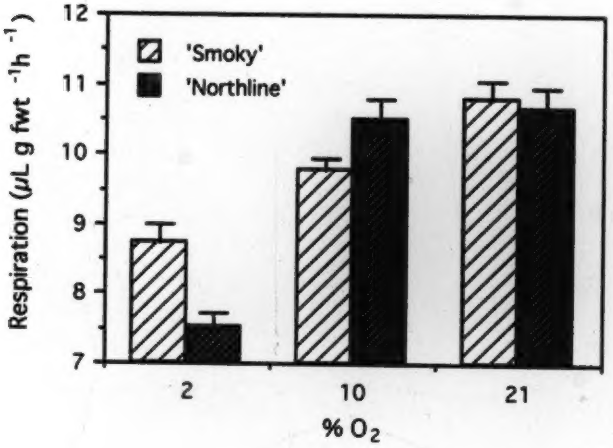


Fig. 6-10. Effects of O_2 atmospheres (balance N_2) on the respiration rates of two cultivars of saskatoon fruit in storage at 4°C. Fruit were ventilated (30 mL min^{-1}) constantly with the appropriate O_2 atmosphere during the 10 day storage interval. Data were averaged over fruit maturity classes (7, 8 and 9) and storage time to show the O_2 concentration x cultivar interaction ($P<0.05$). All other interactions were not significant.

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Chapter VII

Quality of Saskatoon (*Amelanchier alnifolia* Nutt.) Fruit During Low O_2 and Elevated CO_2 Storage

Abstract

Saskatoon fruit were stored under three O_2 levels (2, 10, and 21%) factorially combined with two CO_2 concentrations (0.035% and 5%) and the effects on fruit quality (cvs. Pembina, Smoky, Northline, and Thiessen) were monitored over 56 days at 0.5°C. The 5% CO_2 atmosphere combined with 21 or 10% O_2 was most effective at minimizing losses in fruit soluble solids, anthocyanins, firmness, and fresh weight. Fungal colonization of fruit after 8 weeks of storage was eliminated in 5% CO_2 at all O_2 concentrations. Storage of fruit in 0.035% CO_2 and 21 or 10% O_2 resulted in the highest titratable acidity, lowest pH, and lowest ethanol concentrations; however, ethanol did not exceed 0.03% in fruit stored in any of the atmospheres. Changes in the quality characteristics of fruit during storage in the various CA's were cultivar dependent.

Introduction

Amelanchier alnifolia Nutt. produces a small fruit with a sweet almondy flavor. Fruit are approximately 1.5 cm in diameter and purple when ripe. The fruit is a miniature pome that developes from a gynoecial hypanthium, and is born on shrubs in raceme-like, determinate infructescences containing 8-12 fruit (Steeves and Steeves, 1990; Steeves et al., 1991). In recent years the saskatoon fruit industry has rapidly expanded and currently there are about 1100 ha in production across the prairies of Canada (Hausher, 1996 personal communication). The demand for saskatoon fruit exceeds supply (St. Pierre, 1992) with the fruit either consumed fresh, mainly from 'U-pick' operations, or machine-harvested and processed into value-added products. Although there is current demand for saskatoons as a fresh product at the retail level, lack of adequate transport, storage, and packaging systems for maintaining quality of this highly perishable fruit have impeded development of a fresh market industry.

Controlled atmospheres (CA) can extend shelf-life of produce through delaying ripening, senescence and microbial activity. Both high CO_2 and low O_2 atmospheres maintain postharvest quality by inhibiting respiration, ethylene biosynthesis and ethylene action. An added benefit of these atmospheres is the inhibition of fungal growth by slowing respiration and spore germination (El-Goorani and Sommer, 1981). The optimum concentrations of storage gases are specific for each commodity and cultivar. Exposure of produce to O_2 below, or to CO_2 above commodity-specific tolerance limits may increase anaerobic respiration, resulting in ethanol and acetaldehyde accumulation and off-flavors (Kader et al., 1989). Low O_2 often is better tolerated when CO_2 is maintained close to atmospheric levels (0.035%) because a small increase in CO_2 can have a relatively large inhibitory effect on respiration (Wills et al., 1989). Unfavorable CA conditions can induce physiological breakdown, rendering commodities more susceptible to pathogens (Kader et al., 1989). Storage temperature and duration of exposure to a particular atmosphere can interact to effect quality.

CA treatments were first used to extend the postharvest life of pome fruits approximately 65 years ago (Kidd and West, 1926; 1934), and a majority of the reports on CA have subsequently focused on pome fruits (Kader et al., 1989). Notwithstanding the difficulties inherent in determining the appropriate CA for a commodity, CA's have been developed and are very efficient in prolonging the postharvest life of a diversity of commodities, ranging from fruit to vegetables to flowers. Most cultivars of apple, pear, cherry, and strawberry can tolerate a 2% O_2 atmosphere (Kader et al., 1989), and some varieties of apple can even withstand 1% O_2. Strawberry and other small fruits can tolerate up to 15% CO_2; however, most apple cultivars can only tolerate 5% CO_2 (Kader et al., 1989).

Our previous work has shown good potential for use of subatmospheric O_2 concentrations to maintain saskatoon fruit quality. Ethylene has a central role in regulating the ripening of this climacteric fruit (Rogiers et al., 1997). The rate of ethylene production by saskatoon fruit decreased with declining O_2 concentration, and fruit respiration was also lowered by storage in a 2% O_2 atmosphere (Rogiers and Knowles, 1997b). Other than our work, no information is available on storage systems for this fruit. The objective of this study was to assess the effectiveness of CA storage at maintaining quality of saskatoon fruit. The effects of low O_2 and high CO_2 atmospheres on microbial colonization, fruit firmness, weight loss, titratable acidity, soluble solids, anthocyanins, and ethanol content of fruit were assessed during eight weeks of storage.

Materials and Methods

Fruit Source

Saskatoon fruit (*Amelanchier alnifolia* Nutt. cvs. Pembina, Smoky, Northline and Thiessen) were hand harvested from an orchard in Spruce Grove, Alberta. The fruit were transported on ice and cleaned of debris. Only fruit of maturity class nine (i.e. fully ripe, purple fruit, see Rogiers and Knowles (1997a) for description of maturity classes) were used. Fruit were placed in the appropriate storages (see below) within 5 hours after harvest.

Storage System

Fruit were packaged in 500 mL plastic bottles (150 g/bottle) that were fitted with inlet and outlet ports. The fruit were stored at 0.5°C under a continuous flow (30 ml min^{-1}) of any one of six atmospheres for up to 56 days. Atmospheres consisted of three O_2 concentrations: 2, 10 and 21%; these were factorially combined with CO_2 concentrations of 0.035% and 5%. The atmospheres were formulated and maintained with gas proportioners (Matheson, Secaucus, NJ) from compressed O_2 and CO_2 cylinders, along with a molecular sieve-type nitrogen generator (Permea Inc., St. Louis, Missouri). The atmospheres were humidified (>95% RH) and admitted to the appropriate chambers through manifolds. There were three replicate samples per harvest date for each cultivar, and harvest order was determined at the beginning of the study. Fruit were harvested at 10-20 day intervals throughout the study. Fruit firmness, weight loss and fungal decay were assessed at harvest, and 100 g of fruit from each treatment were then frozen at -30°C for subsequent analysis of soluble solids, anthocyanins, titratable acidity and ethanol.

Quality Analysis

Fungal colonization of fruit was assessed visually. Fruit were rated as 0%, 1-30%, 31-60%, or 61-100% infected. Analysis was based on the midpoint of each category. Fruit firmness was measured with an Instron Universal Testing Machine, model 4201 (Instron Corp., Canton, MA) equipped with a 50 kg load cell. The downspeed of the crosshead was 150 mm min^{-1}. A fruit sample (15 g) was placed in a Kramer Shear Compression Cell (Instron Corp.), adjusted by the insertion of two metal plates so that the bottom area was 14 cm^2 (Gill et al., 1979). The return gauge was set so that the compression cycle terminated as the blades emerged from the bottom of the test cell. Fruit firmness was expressed as the force required to penetrate the sample in kg g^{-1} fresh weight. Firmness was measured for cv. Pembina and Smoky fruit only.

To determine soluble solids, frozen fruit (5 g) were macerated with a polytron (Brinkmann, Rexdale, Ont.) at room temperature and extracts were passed through miracloth. The filtrates were centrifuged for 20 min (1640 g) and an ATAGO hand refractometer, model 500 (ATAGO Co., Ltd., Tokyo, Japan) was used to quantify the refractive index of each supernatant. The refractometer was calibrated with 0% and 10% sucrose solutions, and refractive index values were adjusted to 20°C.

Titratable acidity and pH were analyzed by the electrode method (AOAC, 1995). Frozen fruit (10 g) were extracted at room temperature with 25 mL water using a polytron (1 min). The extracts were centrifuged at 14,500 g (20 min), and pH of the supernatants were recorded. Titratable acidity was measured by titrating the supernatants to pH 8.1 with 0.1 N NaOH, and was expressed as percent malate equivalents.

Anthocyanins were extracted from 5 g of frozen fruit with 10 mL of 95% (v/v) ethanol:1.5 M HCl (85:15 v/v) at 4°C using a polytron. Extracts were centrifuged at 14,500 g for 15 min. and pellets were washed twice with 5 mL of the extraction medium. The supernatants were combined and diluted to 25 mL with extraction medium. Anthocyanins were assayed by the pH differential method of Fuleki and Francis (1968). Extract (100 µL) was added to 2.4 mL of 0.2 N KCl:0.2 N HCl (25:67) buffer (pH 1.0). In addition, 200 µL of extract was combined with 800 µL of 1.0 N sodium acetate:1.0 N HCl:H_2O (100:60:90) buffer (pH 4.5). The samples were equilibrated in the dark for 2 h and A_{510} was recorded. The absorbance at pH 1.0 was subtracted from that at pH 4.5 and total anthocyanins were calculated using the extinction coefficient for cyanidin-3-galactoside ($\varepsilon^{1\%}$ 510 nm = 765), the major anthocyanin of saskatoon fruit (Mazza, 1986).

Ethanol content of 'Thiessen', 'Smoky' and 'Northline' fruit was quantified at 0, 11, and 56 days of storage. No changes in ethanol content were apparent for any of the cultivars between 11 and 56 days. Therefore, only data at 0 and 56 days are reported. Ethanol was extracted from frozen fruit (5 g) with TCA (10% w/v final concentration) at 23°C using a polytron. The crude extracts were transfered to 15 mL test tubes and the tubes were capped with rubber septa. Extracts were then placed at 24°C for 120 min, during which time ethanol equilibrated in the headspace. A 100 µL sample of headspace gas was analyzed for ethanol. Ethanol was separated and quantified on a GC (Varian, Model 3700) equipped with a flame ionization detector and a SPB-5 Supelco column (3 µm film thickness, 30 m x 0.53 mm i.d.) at 40°C. The injector and detector temperatures were 210°C. The carrier gas was He at 40 cm sec^{-1}.

Results

Changes in fungal colonization of maturity class nine fruit over a 56 day storage period at 0.5°C were affected by a cultivar x CO_2 x time interaction. Data were thus averaged across O_2 concentrations. Storage of fruit at 5% CO_2 was more effective than 0.035% CO_2 at slowing pathological deterioration (Fig. 7-1). There were no signs of fungal colonization over the first 11 days of storage in either CO_2 atmosphere. After 56 days of storage at 5% CO_2, only 'Pembina' fruit showed a small amount of infection. The 5% CO_2 atmosphere was effective at inhibiting fungal colonization of 'Smoky' fruit through at least 77 days of storage (Fig. 7-2). Infection of fruit at 0.035% CO_2 was more substantial and depended on cultivar (Fig. 7-1). The rate of colonization was greatest for 'Thiessen', followed by 'Pembina', 'Northline' and 'Smoky' fruit at 0.035% CO_2. The predominant fungal pathogen on fruit was grey mold (*Botrytis cinerea* Pers.).

Changes in percent fresh weight loss of fruit over the 56 day storage period were dictated by CO_2 x O_2 x time, cultivar x time, and cultivar x CO_2 x O_2 interactions. Fruit fresh weight loss during storage never exceeded 3%. When fruit were stored in atmospheres of 10 or 21% O_2, weight loss was higher at 0.035% CO_2 than at 5% CO_2 (Fig. 7-3). However, CO_2 had no effect on weight loss of fruit stored under 2% O_2. Fruit weight loss increased linearly with decreasing O_2 concentration at 5% CO_2 at all storage periods. At 0.035% CO_2, effects of O_2 concentration on weight loss were not apparent until after 22 days. From 33 to 56 days, fruit held at 10 and 21% O_2 maintained higher fresh weights than those stored at 2% O_2.

Unlike 'Thiessen' and 'Northline' fruit, 'Smoky' and 'Pembina' fruit did not lose weight over the first 11 days of storage (Fig. 7-4). By the 56th day of storage, however, 'Pembina', 'Northline' and 'Thiessen' fruit had lost more weight than 'Smoky' fruit. While trends in fresh weight loss with increasing O_2 concentration at 0.035% CO_2 were variable depending on cultivar, a linear decline in fresh weight loss occurred for fruit of all cultivars as O_2 increased at 5% CO_2 (Fig. 7-5). The best storage atmosphere for preserving fruit fresh weight for all cultivars was 21% O_2 combined with 5% CO_2.

Postharvest changes in fruit firmness were characterized by CO_2 x O_2 x time, cultivar x CO_2 x time and cultivar x CO_2 x O_2 interactions. On average, fruit softening was less extensive during storage at 5% CO_2 than at 0.035% CO_2 (Figs. 7-6 and 7-7). Declines in fruit firmness were not evident until after 11 days in any of the controlled atmospheres (Fig. 7-6). At 5% CO_2, the 10% and 21% O_2 atmospheres were most effective at inhibiting softening, with only an average of 7% loss in fruit firmness by the 56th day of storage. However, at 0.035% CO_2, the 2% O_2 atmosphere was most effective at delaying softening, with fruit losing 18% of their firmness after 56 days. Fruit stored at 0.035% CO_2 and 21% O_2 experienced the greatest rate of softening, losing 40% of their firmness over the 56 day interval. From 11 to 56 days of storage at 0.035% CO_2, 'Pembina' fruit softened more rapidly than 'Smoky' fruit; however, the reverse was true at 5% CO_2 (Fig. 7-7A). Trends in fruit softening with increasing O_2 depended on cultivar and CO_2 concentration of the storage atmosphere. Fruit firmness declined linearly with increasing O_2 at 0.035% CO_2, and the rate of decline was more rapid for 'Pembina' than for 'Smoky' fruit (7-7B). In contrast, fruit of both cultivars were the firmest when stored in a 5% CO_2 and 10% O_2 atmosphere. Unlike 'Pembina' fruit, softening of 'Smoky' fruit was relatively insensitive to O_2 when stored in 5% CO_2.

Changes in fruit soluble solids during storage were characterized by CO_2 x O_2 x time and cultivar x time interactions. Similar to fruit firmness, soluble solids content was maintained higher at 5% CO_2 than at 0.035% CO_2 over the 56 day storage period (Fig. 7-8). At 5% CO_2, a 10% O_2 atmosphere was the most effective at inhibiting loss of soluble solids through 33 days of storage. Soluble solids content of fruit from the three O_2 atmospheres was about equal after 56 days of storage at 5% CO_2. At 0.035% CO_2, fruit stored in 2% O_2 maintained higher soluble solids than fruit stored at 10% and 21% O_2 through 33 days. Soluble solids content then declined substantially in all O_2 atmospheres and by the 56th day of storage under low CO_2, fruit soluble solids were the lowest of all treatments. Soluble solids content of 'Pembina' fruit was 8% lower than the combined average for 'Northline', 'Smoky', and 'Thiessen' fruit at the outset of the experiment, and declined 60% faster (based on linear regression) than that of the other cultivars (Fig. 7-9). Soluble solids content of 'Pembina' fruit fell 27%, compared with 13% for the other cultivars, over the 56 day storage period.

Changes in anthocyanin content of fruit over the 56 day storage period were dictated by cultivar x CO_2 x time and cultivar x O_2 interactions. 'Northline' and 'Smoky' fruit had 45% higher anthocyanin concentrations than 'Pembina' and 'Thiessen' fruit at harvest (zero time), and maintained higher concentrations throughout the 56 day storage period (Fig. 7-10). A substantial decline in anthocyanins occurred in fruit of all cultivars during the initial 11 days of storage.

Anthocyanin concentrations and trends in anthocyanin degradation within each cultivar were not greatly affected by CO_2 atmosphere through 33 days of storage. However, from 33 to 56 days in 0.035% CO_2, anthocyanin content of 'Northline', 'Pembina' and 'Thiessen' fruit fell precipitously. Anthocyanin degradation after 33 days of storage was less extensive in 5% CO_2 than in 0.035% CO_2 for fruit of all cultivars. Compared with the other cultivars, anthocyanin content of 'Smoky' fruit declined the least on a percentage basis over the 56 day storage interval.

The effects of O_2 atmospheres on fruit anthocyanins were apparent when data were averaged over time and CO_2 level. Anthocyanin content of 'Northline' fruit declined linearly as O_2 concentration in the storage decreased (Fig. 7-11). Anthocyanin concentrations in fruit of the other cultivars were highest when stored in 10% O_2.

Changes in titratable acidity of fruit during storage were affected by a cultivar x CO_2 x time interaction. Single degree-of-freedom contrasts indicated that the cultivar effects were due to the combinations of 'Northline' and 'Thiessen' versus 'Pembina' and 'Smoky'. Data for the cultivars were thus averaged accordingly. At harvest, fruit titratable acidity was 5.8-fold higher for 'Northline' and 'Thiessen' fruit than for 'Pembina' and 'Smoky' fruit (Fig. 7-12). Titratable acidity of 'Northline' and 'Thiessen' fruit declined by 60% over 56 days of storage and trends were not influenced by CO_2 atmospheres. In contrast, titratable acidity of 'Pembina' and 'Smoky' fruit decreased by 10% in 0.035% CO_2 and by 30% in 5% CO_2. Despite the greater losses of acidity from 'Northline' and 'Thiessen' fruit during storage, fruit of these two cultivars had 2.5-fold greater acidity than 'Pembina' and 'Smoky' fruit after 56 days of storage. Storage CO_2 level also interacted with O_2 concentration to affect fruit acidity (Fig. 7-13). Fruit titratable acidity was lower in all O_2 atmospheres at 5% CO_2, as compared with that at 0.035% CO_2. Moreover, when fruit were stored in 5% CO_2, acidity declined as O_2 concentration increased to 21%. Under 0.035% CO_2, acidity was highest when fruit were stored in 10% or 21% O_2. The most favorable storage atmosphere for high fruit acidity was 10% O_2 combined with 0.035% CO_2.

Postharvest changes in pH of fruit during storage were characterized by CO_2 x O_2 x time and cultivar x time interactions. At 0.035% CO_2, pH of fruit increased from 0 to 22 days of storage, then fell by 0.26 units in 2% O_2, 0.33 units in 10% O_2, and 0.46 units in 21% O_2 (Fig. 7-14). At 5% CO_2, pH of fruit also increased from 0 to 22 days of storage; however, the increases were 0.1 to 0.3 pH units more than that in low CO_2, and the subsequent decline through 56 days was no more than 0.13 units. Fruit pH thus increased more in the 5% CO_2 atmosphere than in the 0.035% CO_2 atmosphere and, in the higher CO_2 atmosphere, pH was relatively insensitive to O_2 level. At harvest, pH of 'Smoky' fruit was 0.66 units higher than the lowest pH cultivar, 'Thiessen' (pH 3.91) (Fig. 7-15). 'Smoky' fruit pH increased with time to a maximum of 4.87 units at 33 days. The maxima in pH of 'Pembina', 'Northline', and 'Thiessen' fruit occurred at 22, 11, and 22 days of storage, respectively.

Changes in fruit ethanol content during storage were defined by CO_2 x O_2 and cultivar x time interactions. Ethanol concentrations in fruit stored at 5% CO_2 were higher than in those stored in 0.035% CO_2, and levels increased with declining O_2 concentration (Fig. 7-16A). In the low CO_2 atmosphere, the greatest increase in ethanol content (1.8-fold) occurred as O_2 declined from 10% to 2%. In contrast, ethanol content increased linearly with declining O_2 concentration in the high CO_2 atmosphere. For all cultivars, fruit ethanol levels increased to a maximum at 11 days of storage (data not shown) and then remained constant through 56 days. The increase in ethanol during storage was greatest in 'Thiessen' fruit, followed by 'Smoky' and 'Northline' fruit (Fig. 7-16B). Acetaldehyde was not detected in fruit stored in any of the controlled atmospheres.

Discussion

Many climacteric fruits (e.g. apple, banana, tomato) are harvested after attaining maximum size and before ripening (ie. at physiological maturity). This practise allows greater time for transport and results in extended storage and shelf life. Even though saskatoon fruit are climacteric and capable of ripening after harvest (Rogiers et al., 1997), fully ripe fruit were used in this study. The large gains in fresh and dry weights (2-fold) of saskatoon fruit throughout the ripening stages of development (maturity classes five through nine) (Rogiers and Knowles, 1997a) negate any benefits that may be obtained by harvesting fruit prior to full ripeness. Storage and packaging systems for saskatoons must thus be designed to preserve quality of the fully ripe fruit.

Previous studies indicated that quality of ripe saskatoon fruit was maintained better at 0.5°C than at 4.0°C over a 40 day storage period (Rogiers and Knowles, 1997b). A 2% O_2 atmosphere was also effective at reducing fruit respiration and ethylene production, suggesting that CA's may further attenuate the postharvest deterioration of saskatoons. One of the most dramatic effects of CA storage on saskatoon fruit quality was the suppression of fungal colonization by high CO_2 (Figs. 7-1, 7-2). Others have established that CO_2 above 10% is needed to significantly suppress fungal colonization (El-Goorani and Sommer, 1981). In this study, fungal colonization of fruit was virtually eliminated during 56 days of storage at 0.5°C in a 5% CO_2 atmosphere (Fig. 7-1) and the effect was independent of O_2 concentration. Under ambient CO_2, lowering the O_2 concentration to 10% or 2% was not effective at delaying fungal infection, and this is consistent with other studies which report that less than 1% O_2 is required to inhibit fungal growth (El-Goorani and Sommer, 1981). Such a low O_2 concentration would not be beneficial to fruit quality due to the likelihood of invoking anaerobic metabolism and the associated development of off-flavors.

Storage in reduced O_2 at 0.035% CO_2 was somewhat effective at maintaining fruit fresh weight (10% O_2, Fig. 7-3), titratable acidity (10% O_2, Fig. 7-13), fruit firmness (2% O_2, Fig. 7-6), and soluble solids content (2% O_2, Fig. 7-8). However 5% CO_2 with either 10% or 21% O_2 was even more effective at maintaining fresh weight, firmness, and soluble solids. Extended quality of saskatoon fruit was less apparent in the high CO_2 / low O_2 atmosphere. In many commodities, tolerance to elevated CO_2 decreases with declining O_2 level (Kader et al., 1989). However, 10% O_2 combined with 5% CO_2 was well tolerated by saskatoon fruit. The 5% CO_2 / 10% O_2 atmosphere was most effective for maintaining high soluble solids and anthocyanin concentrations.

Many of the physiological benefits of storage in high CO_2 are thought to be mediated through effects on ethylene (Sisler and Wood, 1988). Continuous exposure of fruit to high CO_2 affects a decrease in ethylene production within hours or days (Cheverry et al., 1988; Kerbel et al., 1988). High CO_2 reduced ethylene production by kiwi fruit (Rothan and Nicolas, 1994), and the authors suggested that the effect was at least partially manifested by inhibition of the conversion of ACC to ethylene. Carbon dioxide has also been shown to inhibit ethylene action in several systems (Burg and Burg, 1967; Wittenbach and Bukovac, 1973; Abeles and Wydoski, 1987). Ethylene is involved in saskatoon ripening and the production rate is high in maturity class nine fruit (Rogiers et al., 1997). Further studies to determine whether high CO_2 inhibits ethylene production by fully ripe saskatoons, and to what extent this relates to maintenance of postharvest quality, are warranted.

Five percent CO_2 was not as effective as 0.035% CO_2 at maintaining high levels of titratable acidity (Figs. 7-12, 7-13), low pH (Fig. 7-14) or low ethanol (Fig. 7-16) concentrations in fruit. The buildup in ethanol in fruit during storage was likely the result of increased anaerobic metabolism. Ethanol concentrations were higher in fruit stored in reduced O_2 atmospheres and this effect was enhanced in 5% CO_2 (Fig. 7-16). The pasteur point for freshly harvested, ripe saskatoon fruit occurs at less than 2% O_2 under ambient levels of CO_2 (Rogiers and Knowles, 1997). The higher ethanol level of fruit stored in 2% O_2 and 0.035% CO_2 is likely a consequence of prolonged exposure to the atmosphere. The ethanol levels needed to impart off-flavors vary greatly among commodities. An ethanol content of 200 $\mu L\ L^{-1}$ (0.02%) caused a slight off-flavor in '20th Century' pears, but more than 2000 $\mu L\ L^{-1}$ (0.2%) ethanol was required to detect an off-flavor in 'Angelo' plums (Ke et al., 1991). The highest ethanol content recorded for saskatoons in this study was only 300 $\mu L\ L^{-1}$ (0.03%). A sensory evaluation study is needed to determine whether this level of ethanol is sufficient to affect flavor.

In summary, the storage atmospheres that were most effective at maintaining postharvest quality of saskatoon fruit were 5% CO_2 combined with 10% or 21% O_2. While CA-induced changes in fruit quality characteristics varied among cultivars, the cultivar that appeared to maintain quality the longest was 'Smoky'. Further research to assess the residual effects of CA's on the rate of deterioration after removal of fruit from storage is needed.

Fig. 7-1. Changes in fungal colonization of maturity class nine 'Smoky', 'Northline', 'Thiessen', and 'Pembina' saskatoon fruit over 56 days of storage in 0.035 or 5% CO_2 atmospheres at 0.5°C. Fruit were ranked subjectively into any one of four categories; 0%, 1-30%, 31-60%, and 61-100%, based on a visual assessment of fungal colonization. Data analysis was based on the midpoint of each category. The predominant fungal pathogen was grey mold (*Botrytis cinerea* Pers.). The cultivar x CO_2 x time interaction was significant ($P<0.01$). Data is averaged over three O_2 atmospheres.

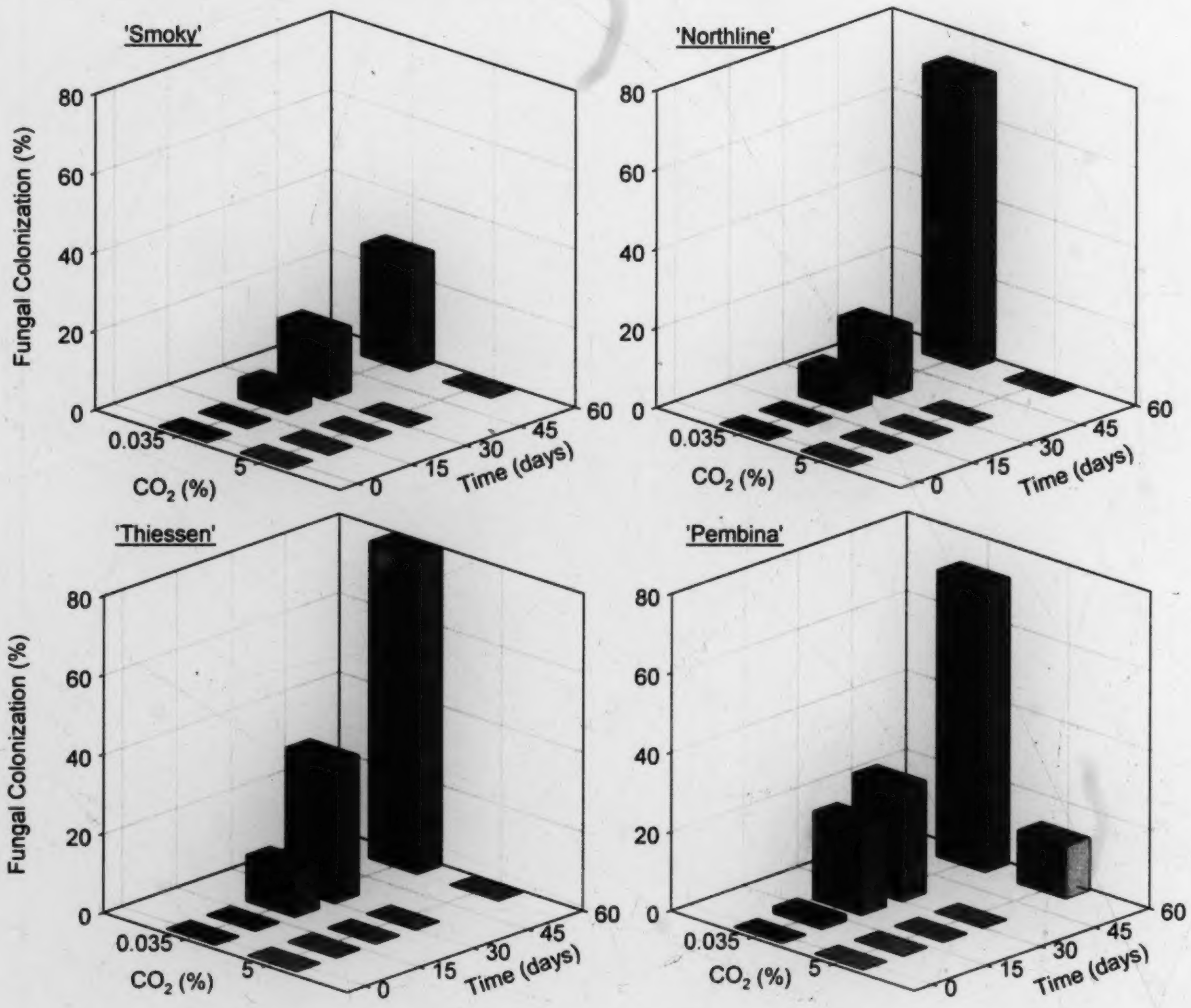
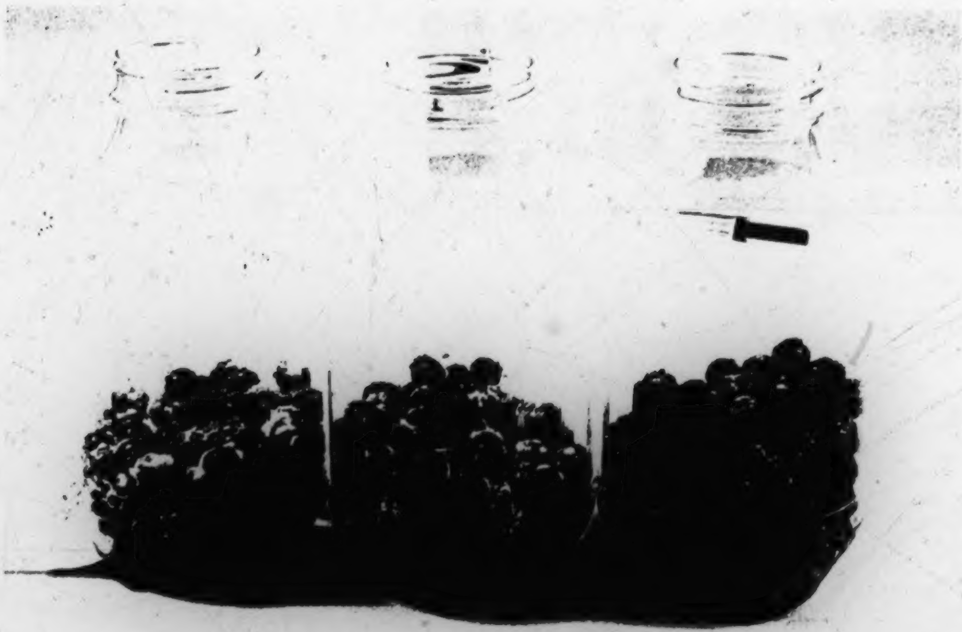

'Smoky'
'Northline'
'Thiessen'
'Pembina'
Fungal Colonization (%)
0
20
40
60
80
0.035
5
CO$_2$ (%)
0
15
30
45
60
Time (days)

Fig. 7-2. 'Smoky' fruit after 77 days of storage at 0.5°C (>95% RH). From left to right, fruit were stored in 21%, 10%, or 2% O_2 atmospheres supplemented with 0.035% CO_2 (A) or 5% CO_2 (B).



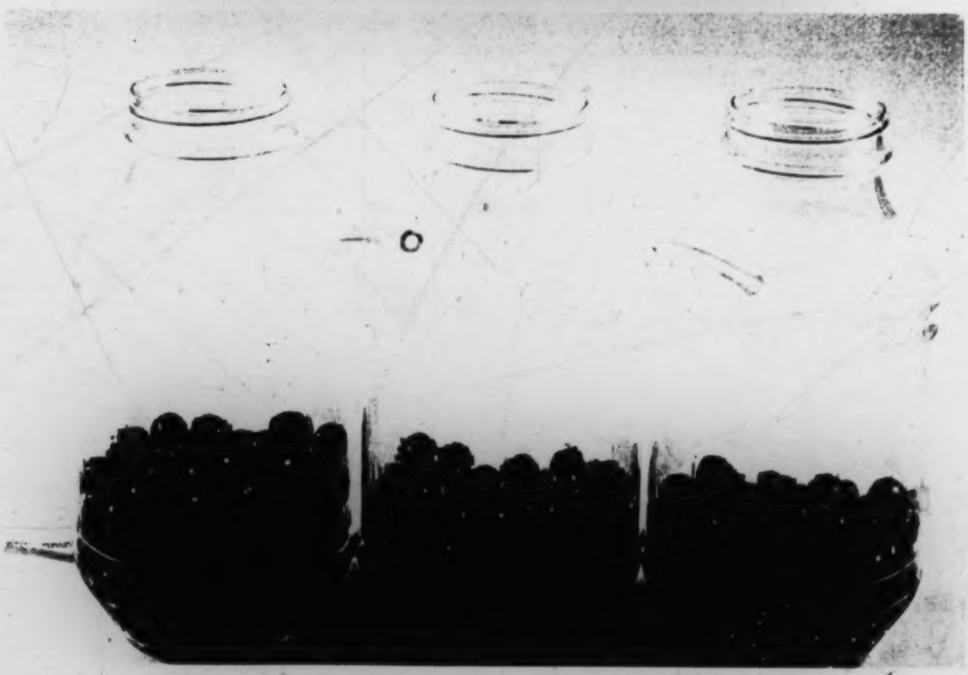
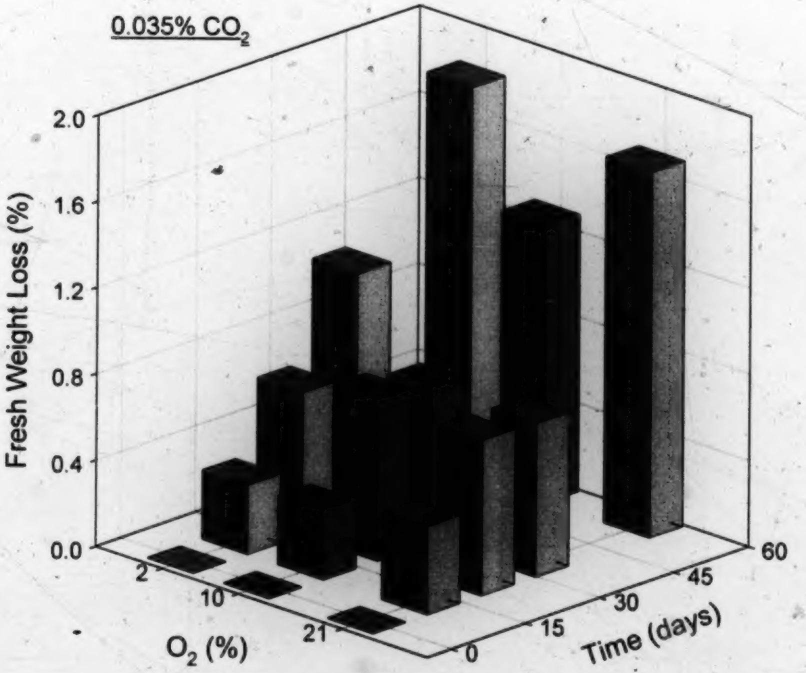
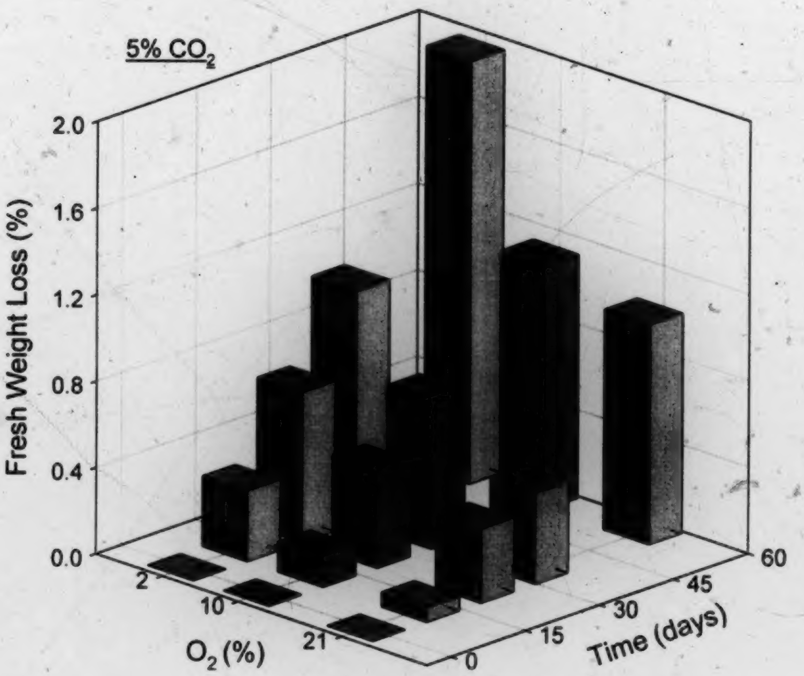


Fig. 7-3. Percent fresh weight loss of maturity class nine saskatoon fruit over 56 days of storage as affected by O_2 and CO_2 atmospheres. Fruit were stored at 0.5°C (>95% RH). The CO_2 x O_2 x time interaction was significant ($P<0.01$). Data is averaged over four cultivars.


0.035% CO$_2$
Fresh Weight Loss (%)
2.0
1.6
1.2
0.8
0.4
0.0
2
10
21
O$_2$ (%)
0
15
30
45
60
Time (days)


5% CO$_2$
Fresh Weight Loss (%)
2.0
1.6
1.2
0.8
0.4
0.0
2
10
21
O$_2$ (%)
0
15
30
45
60
Time (days)

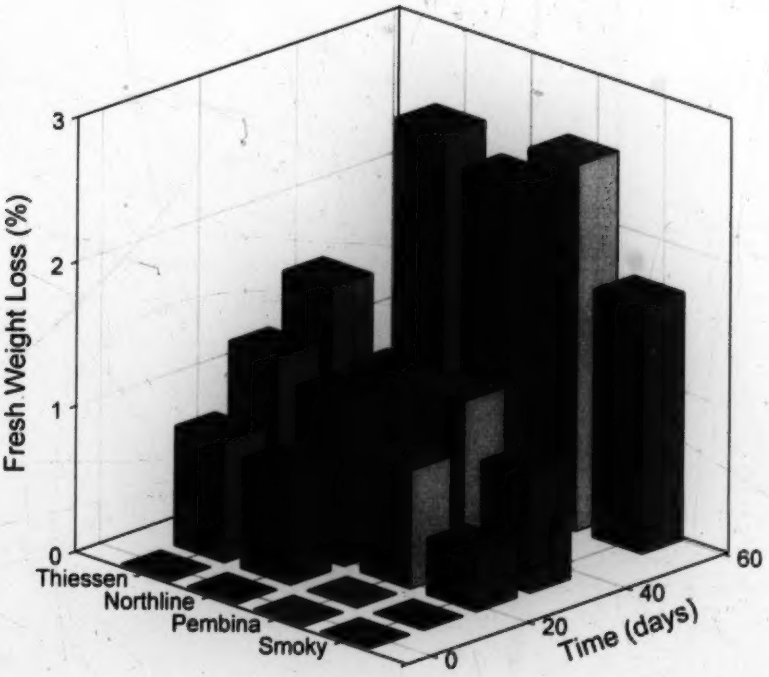


Fig. 7-4. Percent fresh weight loss of maturity class nine 'Thiessen', 'Northline', 'Pembina' and 'Smoky' saskatoon fruit over 56 days of storage at 0.5°C (>95% RH). The cultivar x time interaction was significant ($P<0.01$). Data is averaged over O_2 and CO_2 atmospheres.

Fig. 7-5. Percent fresh weight loss of maturity class nine 'Thiessen', 'Northline', 'Pembina', and 'Smoky' saskatoon fruit during storage at 0.5°C (>95% RH) in various O_2 and CO_2 atmospheres. The cultivar x CO_2 x O_2 interaction was significant ($P<0.05$). Data is averaged over a 56 day storage interval.

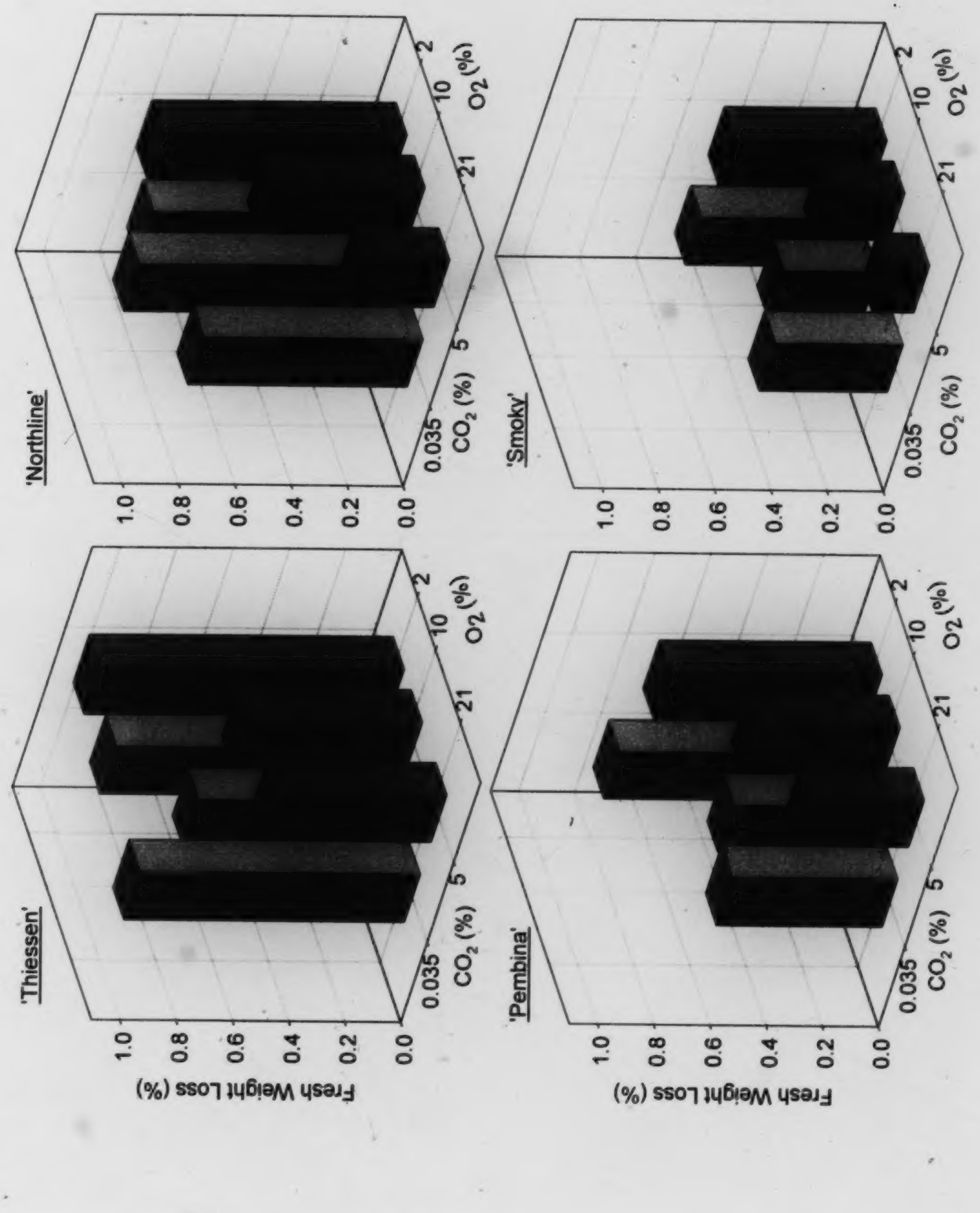
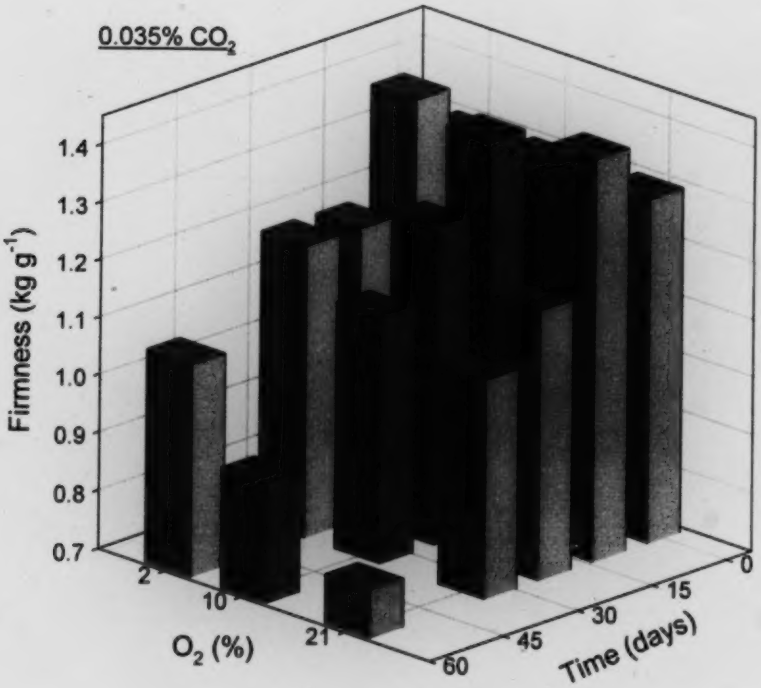

'Thiessen'
'Northline'
'Pembina'
'Smoky'
Fresh Weight Loss (%)
0.0
0.2
0.4
0.6
0.8
1.0
CO$_2$ (%)
0.035
5
O$_2$ (%)
21
10
2

Fig. 7-6. Changes in firmness of maturity class nine saskatoon fruit during 56 days of storage at 0.5°C in various O_2 and CO_2 atmospheres. The CO_2 x O_2 x time interaction was significant ($P< 0.01$). Data is averaged for 'Smoky' and 'Pembina' fruit.


0.035% CO$_2$
Firmness (kg g^{-1})
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
2
10
21
O$_2$ (%)
0
15
30
45
60
Time (days)

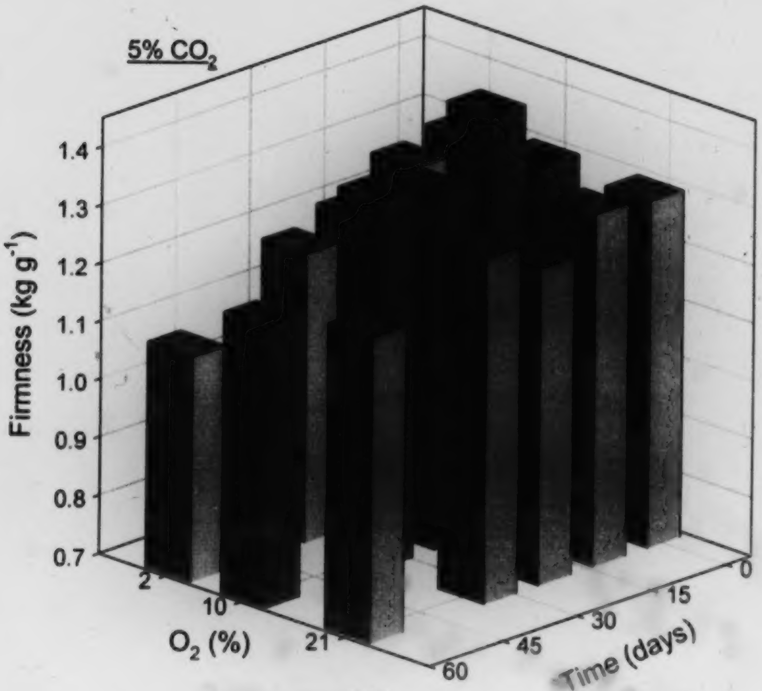
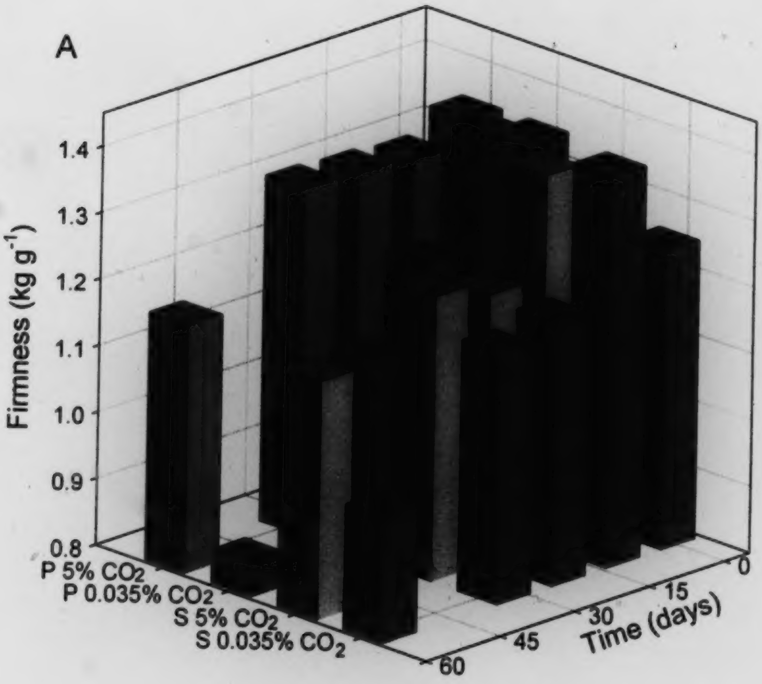

5% CO$_2$
Firmness (kg g^{-1})
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
2
10
21
O$_2$ (%)
0
15
30
45
60
Time (days)

Fig. 7-7. (A) Changes in firmness of maturity class nine 'Pembina' (P) and 'Smoky' (S) saskatoon fruit during 56 days of storage (0.5°C) in 0.035 and 5% CO_2 atmospheres. The cultivar x CO_2 x time interaction was significant ($P<0.01$). Data is averaged over three O_2 atmospheres. (B) Changes in firmness of maturity class nine 'Pembina' (P) and 'Smoky' (S) fruit during storage at 0.5°C, as affected by CO_2 and O_2 atmospheres. The cultivar x CO_2 x O_2 interaction was significant ($P<0.01$). Data is averaged over a 56 day storage interval.


A
Firmness (kg g^{-1})
1.4
1.3
1.2
1.1
1.0
0.9
0.8
P 5% CO2
P 0.035% CO2
S 5% CO2
S 0.035% CO2
Time (days)
0
15
30
45
60

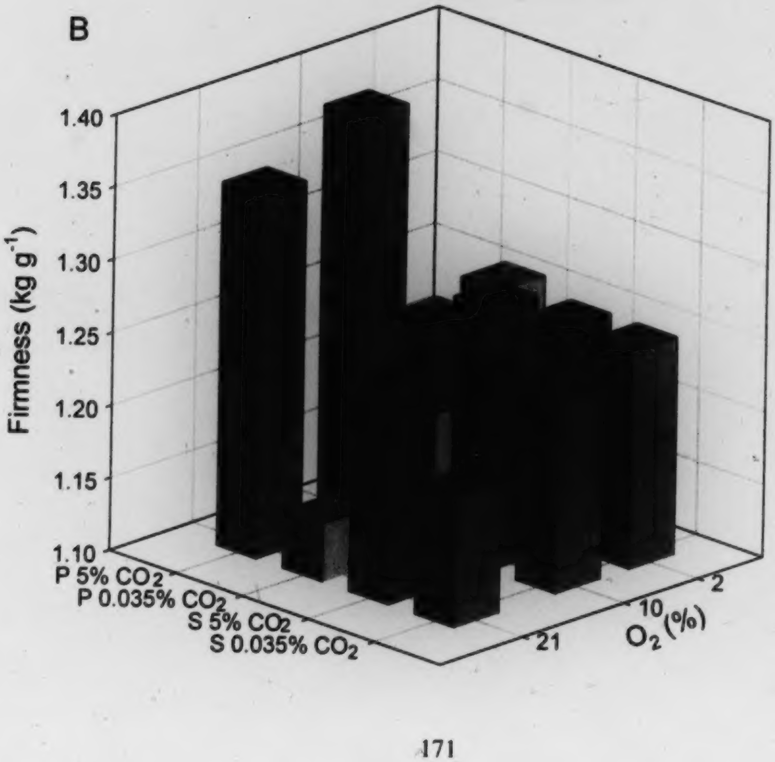
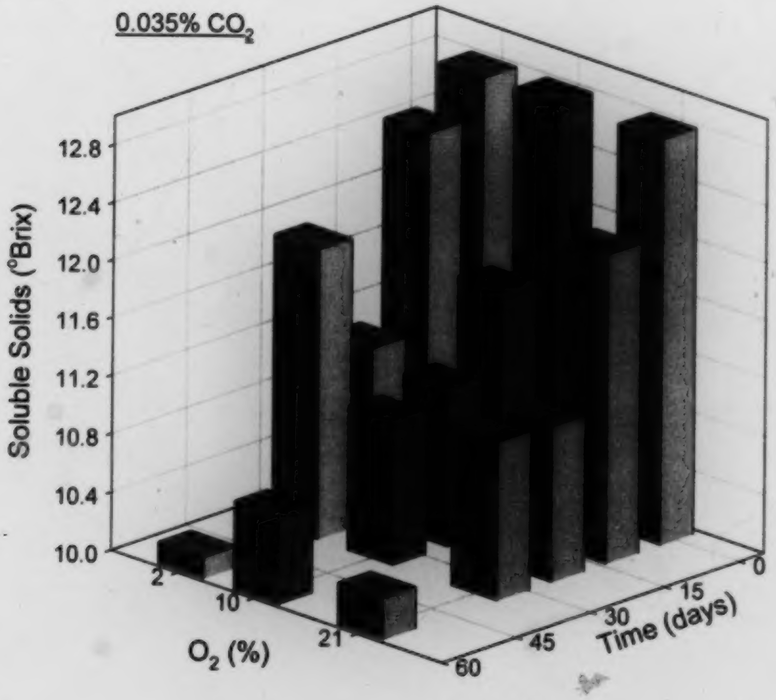
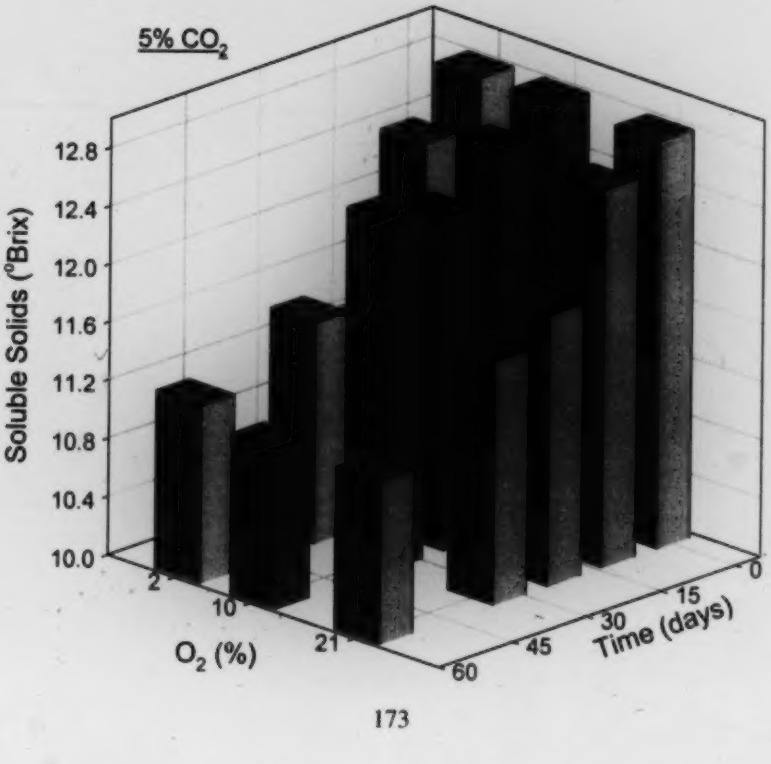

B
Firmness (kg g^{-1})
1.40
1.35
1.30
1.25
1.20
1.15
1.10
P 5% CO2
P 0.035% CO2
S 5% CO2
S 0.035% CO2
O2 (%)
2
10
21

Fig. 7-8. Changes in soluble solids content of maturity class nine saskatoon fruit during 56 days of storage at 0.5°C in various O_2 and CO_2 atmospheres. The CO_2 x O_2 x time interaction was significant ($P< 0.01$). Data is averaged over four cultivars.


0.035% CO$_2$
Soluble Solids (°Brix)
12.8
12.4
12.0
11.6
11.2
10.8
10.4
10.0
2
10
21
O$_2$ (%)
0
15
30
45
60
Time (days)


5% CO$_2$
Soluble Solids (°Brix)
12.8
12.4
12.0
11.6
11.2
10.8
10.4
10.0
2
10
21
O$_2$ (%)
0
15
30
45
60
Time (days)

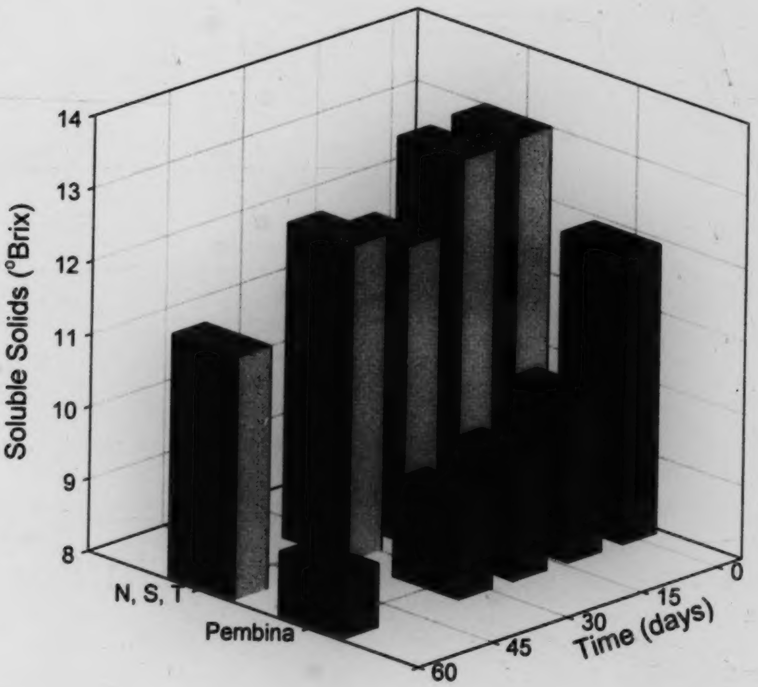
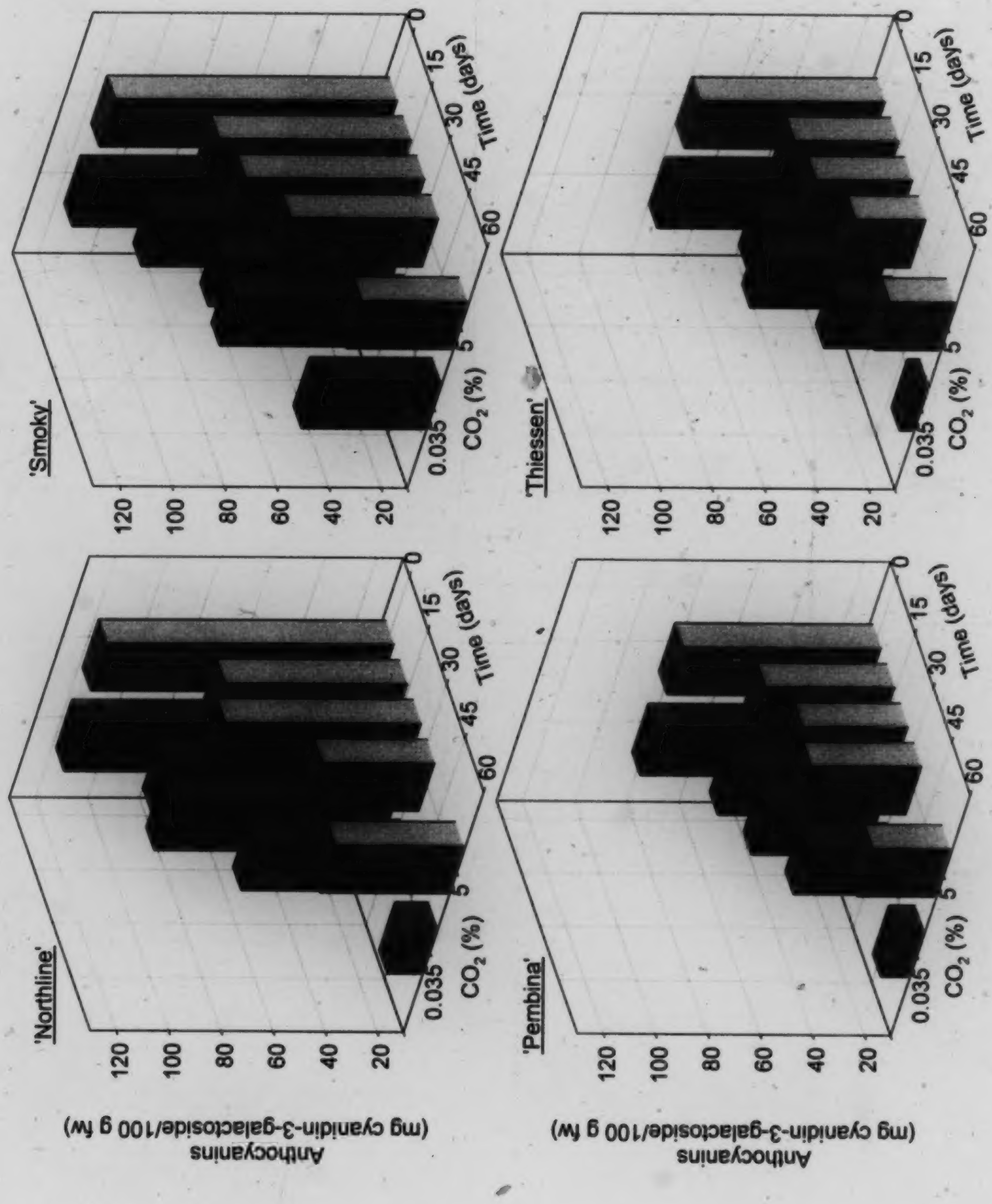


Fig. 7-9. Changes in soluble solids content of maturity class nine saskatoon fruit during 56 days of storage at 0.5°C. The average of 'Northline' (N), 'Smoky' (S), and 'Thiessen' (T) fruit is compared with that for 'Pembina' fruit. The 'Pembina' versus N,S,T x time interaction was significant ($P < 0.01$). Data is averaged over O_2 and CO_2 atmospheres.

Fig. 7-10. Changes in anthocyanin concentrations of maturity class nine 'Northline', 'Smoky', 'Pembina', and 'Thiessen' saskatoon fruit during 56 days of storage in 0.035 and 5% CO_2 atmospheres at 0.5°C. The cultivar x CO_2 x time interaction was significant ($P<0.01$). Data is averaged over three O_2 atmospheres.


'Northline'
'Smoky'
'Pembina'
'Thiessen'
Anthocyanins
(mg cyanidin-3-galactoside/100 g fw)
120
100
80
60
40
20
CO$_2$ (%)
0.035
5
Time (days)
0
15
30
45
60

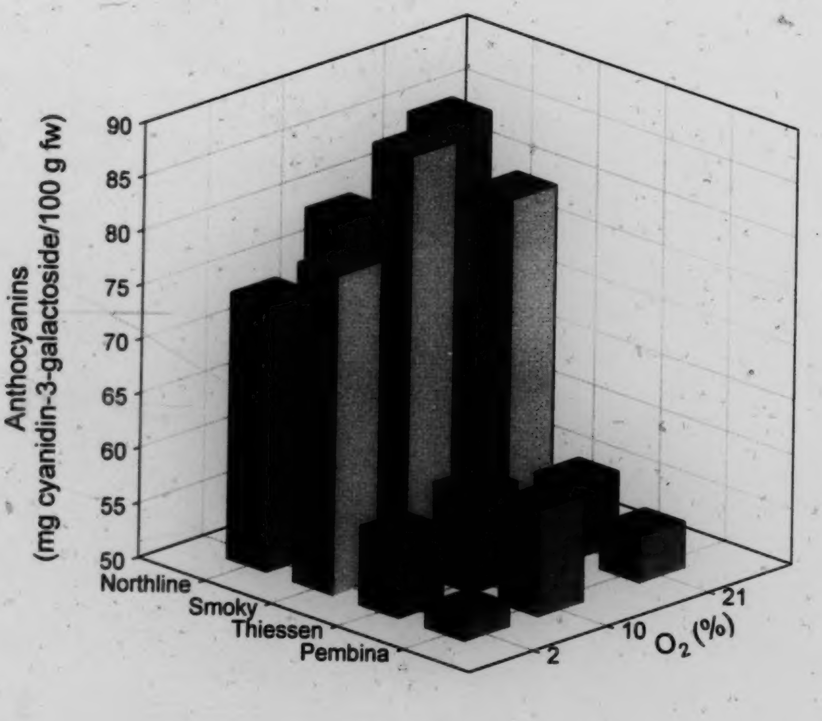
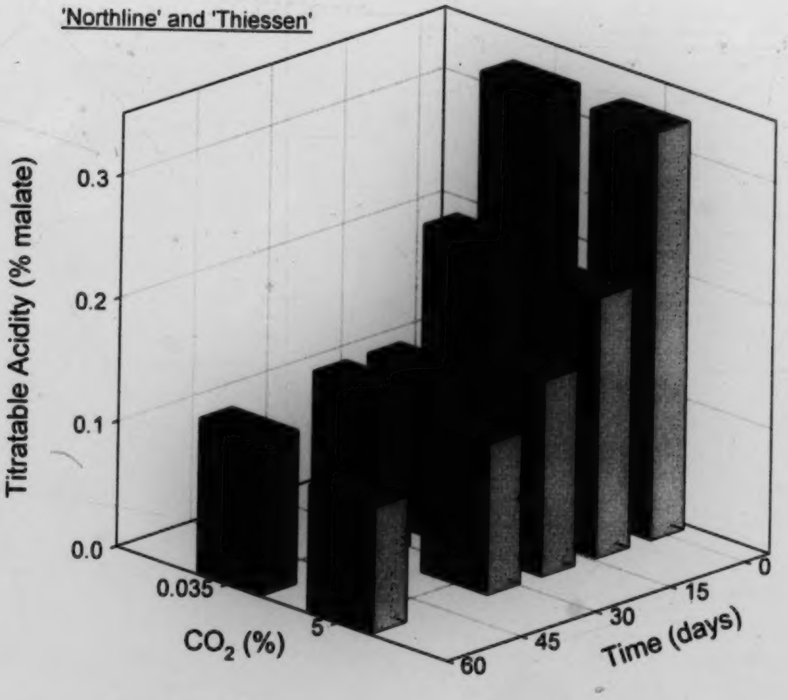
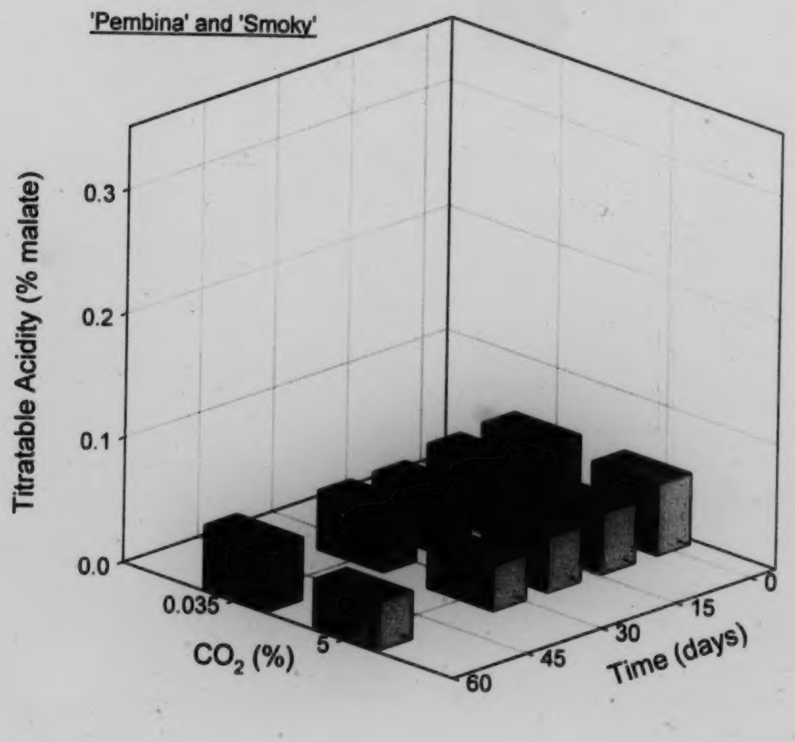


Fig. 7-11. Changes in anthocyanin concentrations of maturity class nine 'Northline', 'Smoky', 'Thiessen' and 'Pembina' fruit as affected by storage in 2, 10, and 21% O_2 atmospheres at 0.5°C. The cultivar x O_2 interaction was significant ($P<0.01$). Data is averaged over two CO_2 atmospheres and a 56 day storage interval.

Fig. 7-12. Changes in titratable acidity of maturity class nine 'Northline', 'Thiessen', 'Pembina' and 'Smoky' fruit during 56 days of storage in 0.035 and 5% CO_2 atmospheres at 0.5°C. The cultivar x CO_2 x time interaction was significant ($P<0.01$). Cultivars were averaged based on results of orthogonal contrasts. Data is expressed as percent (f wt) malate equivalents, the predominant organic acid of mature fruit (Rogiers and Knowles, 1997), and is averaged over three O_2 atmospheres.


'Northline' and 'Thiessen'


'Pembina' and 'Smoky'

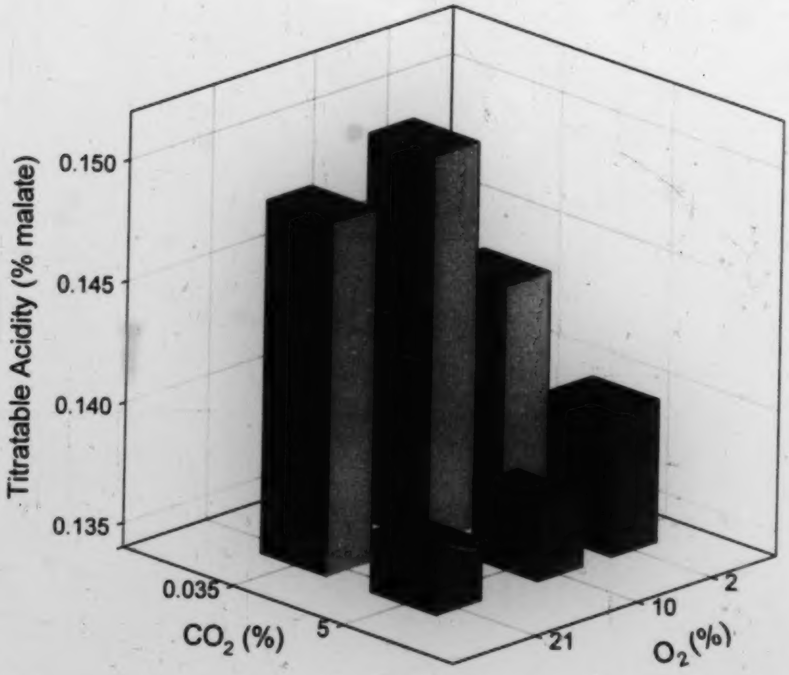
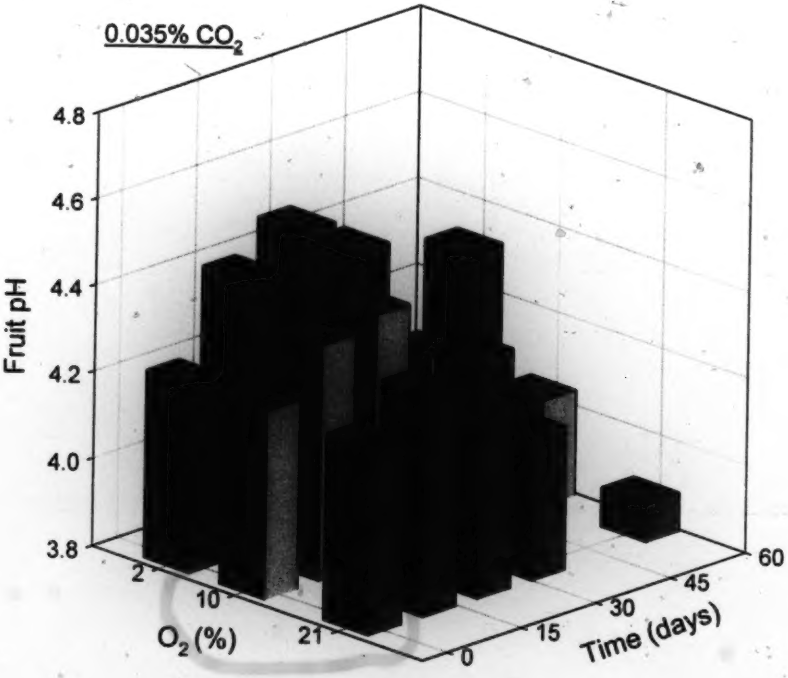
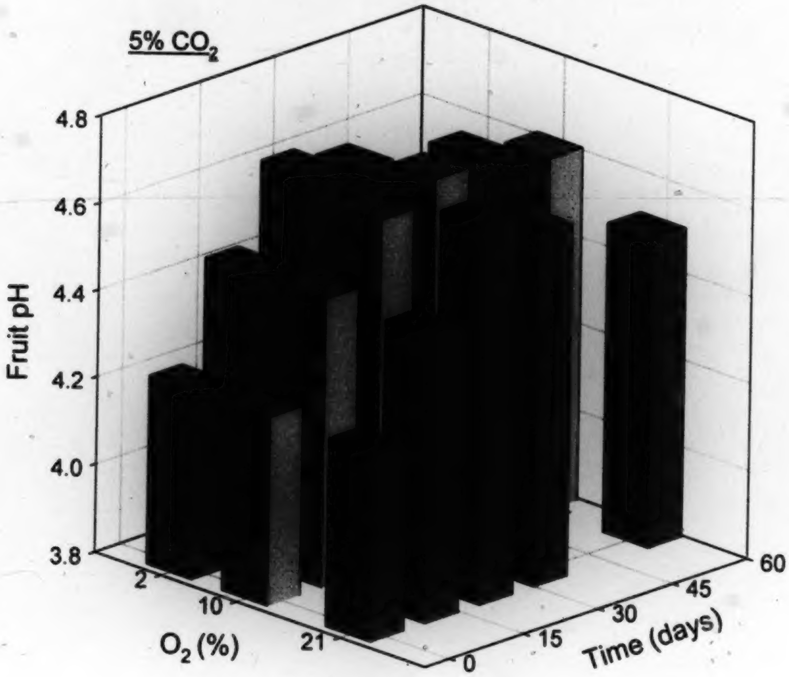


Fig. 7-13. Changes in titratable acidity of maturity class nine saskatoon fruit during 56 days of storage at 0.5°C in various O_2 and CO_2 atmospheres. The CO_2 x O_2 interaction was significant ($P<0.05$). Data is expressed as percent (fwt) malate equivalents, the predominant organic acid of mature fruit (Rogiers and Knowles, 1997), and is averaged over four cultivars and a 56 day storage interval.

Fig. 7-14. Changes in pH of maturity class nine saskatoon fruit during 56 days of storage in various O_2 and CO_2 atmospheres at 0.5°C. The CO_2 x O_2 x time interaction was significant ($P<0.05$). Data is averaged over four cultivars.


0.035% CO_2
Fruit pH
4.8
4.6
4.4
4.2
4.0
3.8
2
10
21
O_2 (%)
0
15
30
45
60
Time (days)


5% CO_2
Fruit pH
4.8
4.6
4.4
4.2
4.0
3.8
2
10
21
O_2 (%)
0
15
30
45
60
Time (days)

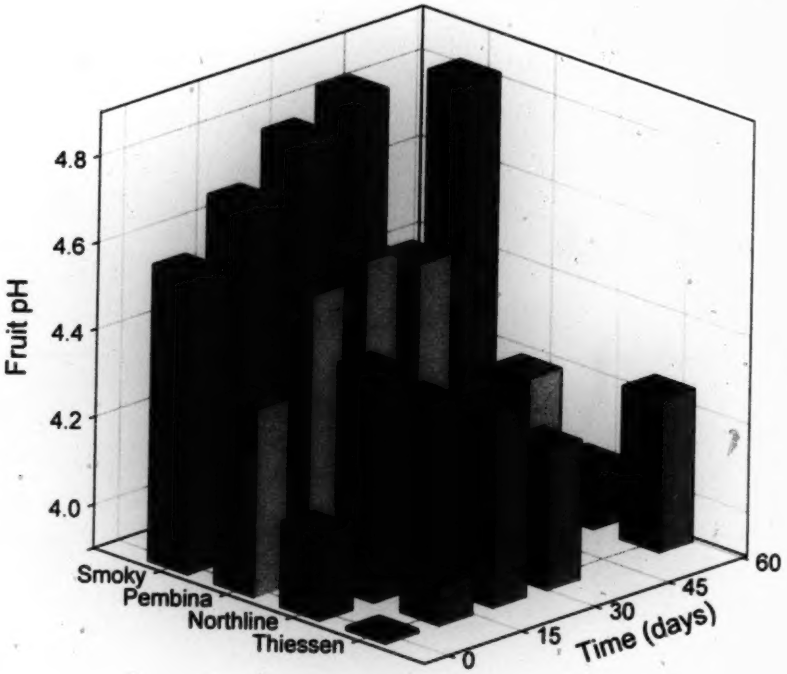
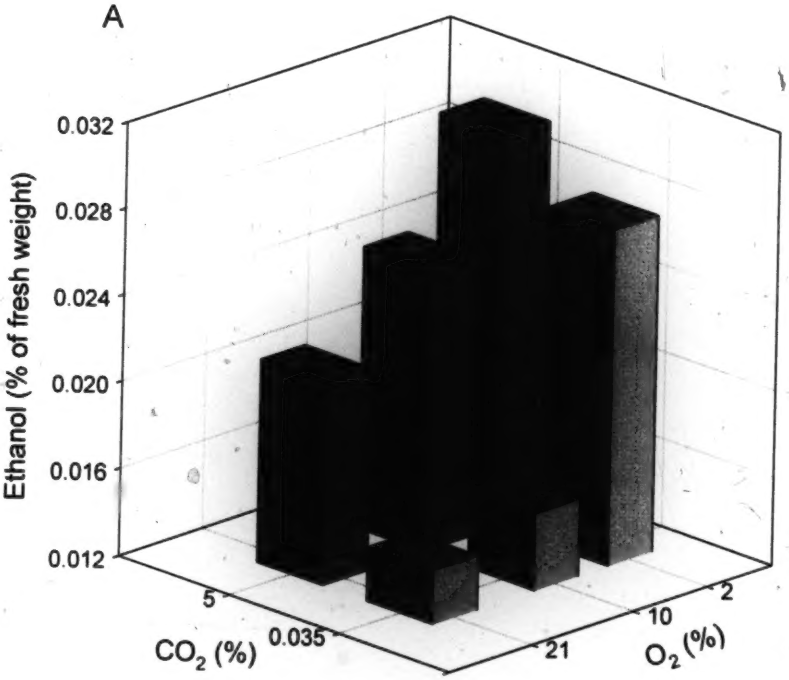


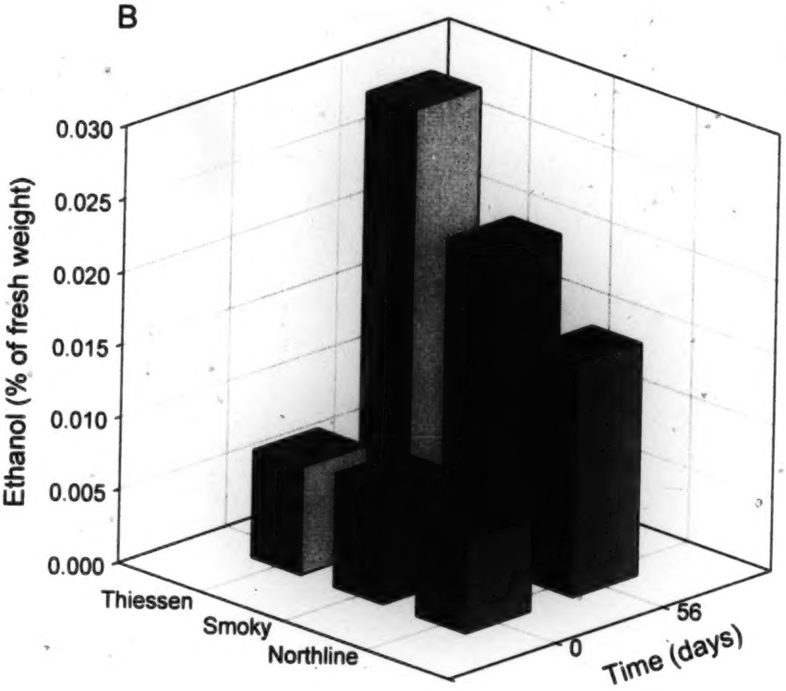
Fig. 7-15. Changes in pH of maturity class nine 'Smoky', 'Pembina', 'Northline' and 'Thiessen' fruit during 56 days of storage at 0.5°C. The cultivar x time interaction was significant ($P<0.01$).Data is averaged over O_2 and CO_2 atmospheres.

Fig. 7-16. (A) Changes in ethanol concentrations of maturity class nine saskatoon fruit during storage at 0.5°C, as affected by CO_2 and O_2 atmospheres. The CO_2 x O_2 interaction was significant ($P<0.01$). Data is averaged over three cultivars and a 56 day storage interval. (B) Ethanol concentrations of maturity class nine 'Thiessen', 'Smoky' and 'Northline' fruit at harvest and after 56 days of storage at 0.5°C. The cultivar x time interaction was significant ($P<0.01$). Data is averaged over O_2 and CO_2 atmospheres.

A


Ethanol (% of fresh weight)
0.032
0.028
0.024
0.020
0.016
0.012
5
0.035
CO$_2$ (%)
2
10
21
O$_2$ (%)

B


Ethanol (% of fresh weight)
0.030
0.025
0.020
0.015
0.010
0.005
0.000
Thiessen
Smoky
Northline
56
0
Time (days)

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Rogiers, S.Y., and N.R. Knowles. 1997b. Effects of storage temperature and atmosphere on saskatoon (*Amelanchier alnifolia* Nutt.) fruit quality, respiration, and ethylene production. (in review)

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Chapter VIII

Summary and Conclusions

With the exception of 'U-pick' and farmer's-market-type sales, most of the commercial saskatoon crop is harvested, frozen and processed into value-added products (jams, jellies, juices, etc.). The development of a viable retail industry for fresh saskatoon fruit is impeded by lack of efficient handling, storage and packaging systems that preserve fresh fruit quality. Understanding the metabolic bases for ripening and subsequent deteriorative changes in harvested fruits is prerequisite to defining storage systems that will best preserve quality. This work has characterized ripening physiology, biochemistry and postharvest behavior of saskatoon fruit. In addition, the efficacy of controlled atmosphere storage for slowing physiological and pathological deterioration was tested.

A physicochemical characterization of the maturation and ripening phases of saskatoon fruit led to the development of a nine stage maturity index. The fastest gains in fresh and dry weight occurred over the latter stages of development. Fruit firmness declined as did pH, chlorophyll, and phenolics. Anthocyanins increased progressively from the fourth stage of development. Glucose and fructose, along with sorbitol increased throughout ripening and were the major carbohydrates in fruit. Succinate was the predominant organic acid in immature fruit, however malate was predominant in ripe fruit. The lack of starch and the high concentrations of reducing sugars are typical of many soft fruit, but are unlike that of other pome fruit. The maturity index system facilitated much of the subsequent work on saskatoons in our lab and has been used successfully by other researchers (McGarry et al., 1997). It is hoped that the industry finds this system useful as an indicator of fruit development.

The saskatoon fruit is climacteric in that ethylene regulates ripening. There is a concomitant increase in endogenous ethylene and ethylene evolution during ripening. Furthermore, ACC application to attached fruit hastened ripening, while inhibitors of ethylene synthesis delayed ripening. Lack of increase in respiration on a fresh weight basis during ripening merely reflects the rapid gain in fresh weight toward the end of development. A respiratory climacteric was evident on a whole-fruit basis concurrent with ripening.

As is characteristic of many other climacteric fruit, physiologically mature saskatoons were able to ripen after harvest. This may have important implications for the processing industry where fruit is machine harvested. Unripe fruit could be induced to ripen after harvest, affecting an overall increase in marketable yield. However, the great increase in size that occurs during the latter stages of development indicates that a higher economic return will likely come from harvesting the greatest amount of fully ripe fruit. In this regard, further research on methods of inducing uniform fruit development and ripening is needed.

Oxidative stress accompanied the physicochemical changes associated with ripening. Lipid peroxidation was indicated by progressive increases in the saturation of membrane lipids, ethane and TBARS during ripening. LOX activity increased while SOD and CAT activities decreased during ripening. These changes likely affected increases in superoxide and lipid hydroperoxides in fruit and thus a greater potential for oxidative catabolism during ripening. Oxidized glutathione increased as a percentage of total glutathione, reflecting the increase in oxidative status of fruits during ripening. It would be of interest to determine the extent to which CA's inhibit oxidative metabolism in maintaining fruit quality during storage.

Storage of fruit at 0.5°C was more effective than storage at 4.0°C for slowing physiological and pathological deterioration. Fungal infection of fruit was absent during 8 weeks of storage at 0.5°C in 5% CO_2. 5% CO_2 with 21 or 10% O_2 atmospheres was most effective at maintaining fruit soluble solids, anthocyanins, firmness, and fresh weight, however the 0.035% CO_2 atmosphere combined with 21 or 10% O_2 best maintained fruit acidity and low ethanol concentrations.

In summary, our studies have:

1. characterized developmental changes in chemical components of fruit such as sugars, organic acids, phenolics and pigments, as well as fruit size, firmness and color. A nine-stage maturity index system based on subjective criteria was linked to the various physicochemical changes associated with fruit development.
2. shown that ethylene is responsible for regulating the ripening of saskatoon fruit.
3. indicated that ripening is accompanied by a progressive increase in oxidative/ peroxidative status of fruit that likely facilitates many of the changes in fruit quality associated with ripening.
4. shown that large increases in fruit size during ripening dictate that these fruit be harvested fully ripe. Therefore, postharvest life is

limited and systems must be developed to preserve quality in fully-ripe fruit.

5. shown that controlled atmosphere storage is very effective at inhibiting fungal growth and extending the postharvest life of saskatoon fruit.